PATENT
Docket No. 240042052403

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office on February 5, 2002.

Rhea Amid

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Jeffrey S. Glenn

Serial No.: 09/687,267

Filing Date: October 13, 2000

For: METHOD FOR INHIBITION OF VIRAL MORPHOGENESIS

Examiner: B. Brumback

Group Art Unit: 1642

DECLARATION OF JEFFREY S. GLENN PURSUANT TO 37 C.F.R. §1.132

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Considered 2-21-02 [illegible]

Dear Sir:

I, Jeffrey S. Glenn, declare as follows:

1. I am the sole inventor of the subject matter claimed in the above-referenced application.

2. I have conducted experiments demonstrating that:

1) the prenylation inhibitors FTI-277 and FTI-2153 can be used to treat hepatitis delta virus (HDV) infection *in vivo*; and

2) FTI-277 and FTI-2153 can effectively inhibit the production of HDV virions at a concentration that is not toxic to the testing animals.

EXHIBIT A
SN 09/687,267

These experimental results are set forth in the following paragraphs 3-4 and in attached Figures 1A-D.

3. HBV-transgenic mice were inoculated by hydrodynamic transfection to initiate authentic HDV genome replication. Mice were treated for one week by IP injection with vehicle alone (Figure 1A and 1B, lanes 1 and 6), vehicle + 50 mg/kg/day FTI-277 (Figure 1A and 1B, lanes 2-5), or vehicle + 50 mg/kg/day FTI-2153 (Figure 1A and 1B, lanes 7-10). Serum samples were then analyzed for HDV virions by RT-PCR analysis (Figure 1A and 1B, lanes 1-10). The primers used in the RT-PCR assay yield a 540 bp fragment only in the presence of circular viral genomic RNA, as found in virions. The production and release of HDV virions into the serum was completely eliminated in the groups treated with prenylation inhibitors.

4. Non-specific toxicity of the FTI-277 and FTI-2153 on the testing animals was assessed by alanine aminotransferase (ALT) assays, which is a standard "liver function" test, (Figure 1C and 1D) performed on aliquots of the corresponding serum samples from Figure 1A and 1B. For the dosages tested, on average, animals treated with FTI-277 have the same level of ALT as the placebo and animals treated with FTI-2153 have lower level of ALT than the placebo.

5. Taken together, the above results demonstrate that the prenylation inhibitors FTI-277 and FTI-2153 can effectively inhibit HDV virion production *in vivo*. This inhibition is not associated with, and cannot be explained by, non-specific toxicity in the testing animals.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Palo Alto, California, on February 5, 2002.

Jeffrey S. Glenn

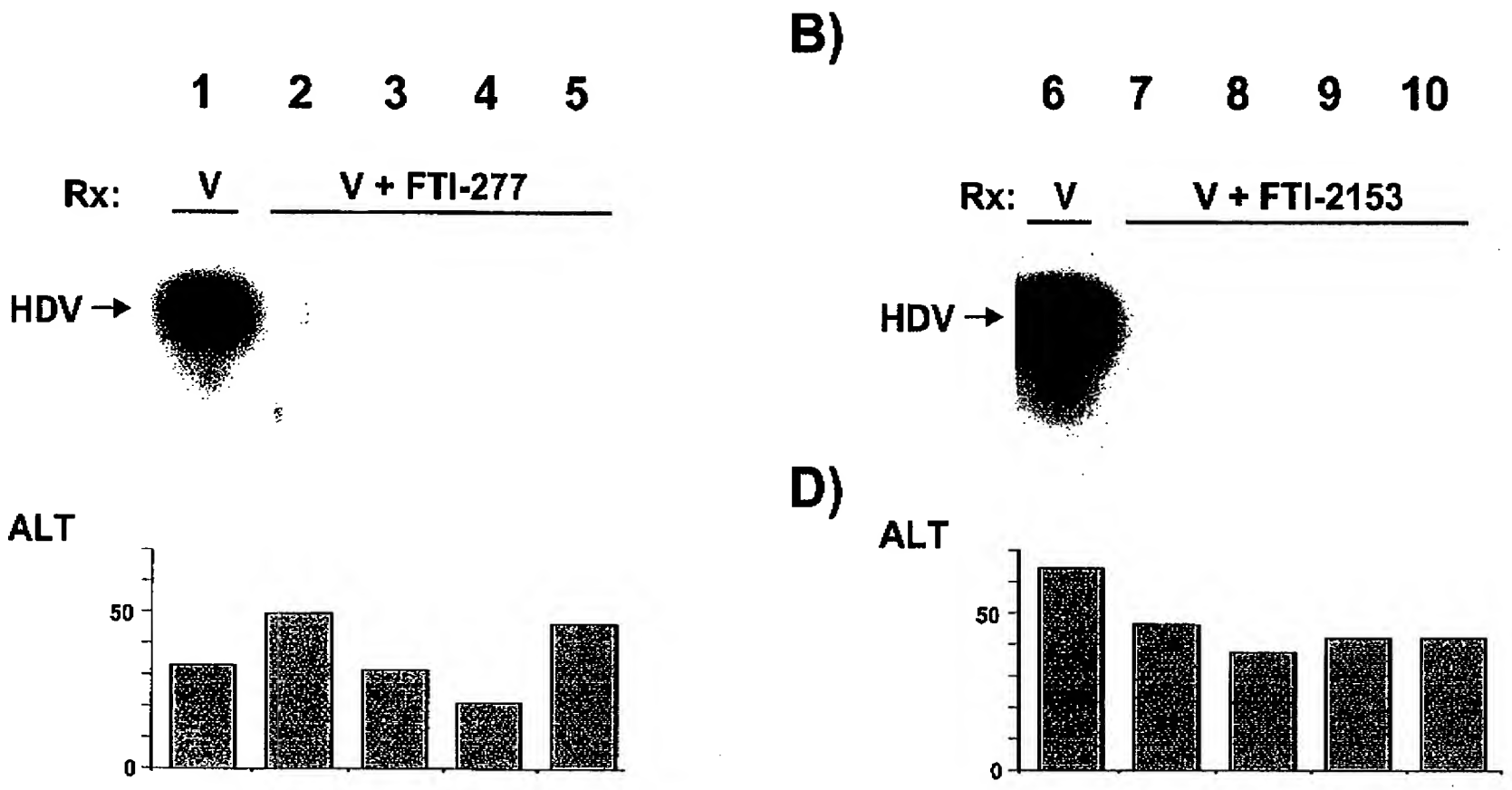

Figure 1. In vivo treatment of hepatitis delta virus (HDV) with the prenylation inhibitors FTI-277 and FTI-2153. HBV-transgenic mice were inoculated by hydrodynamic transfection to initiate authentic HDV genome replication. Mice were treated for one week by IP injection with vehicle alone (lanes 1 and 6), vehicle + 50 mg/kg/day FTI-277 (lanes 2-5), or vehicle + 50 mg/kg/day FTI-2153 (lanes 7-10). Serum samples (lanes 1-10) were then analyzed for HDV virions by RT-PCR analysis (**A** and **B**), and non-specific toxicity by ALT assays (**C** and **D**). The primers used in the RT-PCR assay yield a 540 bp fragment only in the presence of circular viral genomic RNA, as found in virions (**HDV**). Note that the production and release of HDV virions into the serum was completely eliminated in the groups treated with prenylation inhibitors, and no significant toxicity was observed compared to vehicle-treated control animals (See text for details).

2318 Vol. 6, 2318–2325, June 2000 Clinical Cancer Research

Comparison of Potential Markers of Farnesyltransferase Inhibition[1]

Alex A. Adjei,[2] Jenny N. Davis, Charles Erlichman, Phyllis A. Svingen, and Scott H. Kaufmann

Divisions of Medical Oncology [A. A. A., C. E.] and Oncology Research [J. N. D., P. A. S., S. H. K.], Mayo Clinic and Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Graduate School [S. H. K.], Rochester, Minnesota 55905

ABSTRACT

Farnesyltransferase inhibitors (FTIs) were developed to target abnormal signaling pathways that are commonly activated in neoplastic cells. Five FTIs have recently undergone Phase I testing; and two are currently in Phase II clinical trials. As part of the development of these agents, there has been interest in determining their cellular effects in the clinical setting. Several approaches have been proposed, including measurement of FT enzymatic activity, evaluation of the processing of FT polypeptide substrates, and assessment of the accumulation of $p21^{waf1}$. In the present study, a number of these assays have been compared in four cultured human neoplastic cell lines of different histology (A549, HCT116, BxPC-3, and MCF-7) after treatment with the nonpeptidomimetic FTI SCH66336 and the peptidomimetic inhibitor FTI-277. Immunoblotting studies failed to demonstrate a mobility shift in ras proteins or increased accumulation of $p21^{waf1}$ after treatment with these agents. In contrast, drug-induced increases in the slower migrating, unprocessed species of the chaperone protein HDJ-2 and the intranuclear intermediate filament protein lamin A were detected in all four cell lines after treatment with either agent. Unprocessed forms of both polypeptides accumulated in noncycling as well as cycling cells. The precursor peptide that is present in prelamin A but absent from mature lamin A could be readily detected by immunohistochemistry in noncycling cells with a peptide-specific antiserum. Our results indicate that unprocessed HDJ-2 and prelamin A should be suitable markers of FT inhibition in clinical samples.

INTRODUCTION

FT[3] catalyzes the first step in the posttranslational modification of a small number of cellular polypeptides (1, 2). Among the polypeptide substrates of FT are the ras proteins, which are mutated in ~30% of human cancers (3). As a result of early reports indicating that farnesylation was required for maturation of ras proteins, there has been extensive interest in FT as a potential target of antineoplastic therapy (4–8). To date, at least five different FTIs, SCH66336, R115777, L778123, BMS214662, and FTI-277 have entered clinical testing.

Because these compounds are among the first potential anticancer drugs that inhibit aberrantly activated signal transduction pathways in neoplastic cells, there is considerable interest in assessing their cellular and subcellular effects in the clinical setting. Conceptually, assays of FTI action could serve two purposes. In early clinical trials, these assays will be required to determine whether FT has been inhibited at drug concentrations that are achievable in the clinical setting. In later clinical trials, these assays could potentially provide an early marker of drug efficacy if a strong correlation between assay results and clinical outcome can be established. A variety of assays have been proposed for these purposes.

Because the posttranslational processing of ras provided the initial rationale for the clinical development of FTIs (7–10), many earlier studies focused on alterations in ras itself. FT inhibition results in altered mobility of H-ras on SDS-polyacrylamide gels, particularly when H-ras-transfected murine cells are examined (7, 11–14). Unfortunately, the existence of alternative prenylation pathways makes it difficult to detect altered processing of other ras isoforms after FT inhibition in many cells (15, 16). Moreover, a number of different observations have raised the possibility that other FT substrates, notably RhoB, might be involved in the antiproliferative effects of FTIs (4, 10, 17–19). These considerations have prompted investigators to search for other assays to monitor the effects of FTIs.

Conceptually, the simplest assay for FT inhibition involves preparing extracts from FTI-treated cells and measuring the remaining ability of FT to farnesylate a substrate polypeptide. In situations in which the inhibitor dissociates from the enzyme, however, these enzymatic assays are likely to underestimate the degree of FT inhibition. Moreover, these assays are difficult to implement in the setting of multi-institution clinical trials.

Other potential assays evaluate the inhibition of processing of other FT substrates. Several farnesylated polypeptides, including the chaperone protein HDJ-2 (20), the peroxisomal protein Pxf (21), and the intranuclear intermediate filament protein lamin A (22), are known to undergo mobility shifts when FT is inhibited (23, 24). In the case of lamin A, this mobility shift reflects inhibition of proteolytic processing that removes a 13-amino acid peptide from the COOH terminus of prelamin A

Received 1/3/00; revised 3/28/00; accepted 3/30/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by Grants K01 CA77112, U01 CA69912, and P30 CA15083.
[2] To whom requests for reprints should be addressed, at Division of Medical Oncology, Mayo Clinic, 200 First Street, S.W., Rochester, MN 55905. E-mail: Adjei.Alex@Mayo.edu.

[3] The abbreviations used are: FT, farnesyltransferase; FTI, farnesyltransferase inhibitor.

to yield the mature lamin (23). Because this processing is absolutely dependent on farnesylation (25), it has been suggested that the accumulation of prelamin A containing this unique COOH-terminal peptide might be a potential marker of FT inhibition (26).

In addition to these assays, it has been proposed that certain downstream events might also be used to monitor the effects of FTIs. For example, treatment with the FTI L744832 has been shown to cause increases in levels of the cyclin-dependent kinase inhibitor $p21^{waf1}$ in p53 wild-type cells (27). In addition, the activity of downstream kinases such as raf-1, MEK, or ERK1 or the phosphorylation state of MEK and ERK1 could, in principle, be used as a readout of FT inhibition (12, 28–30).

Although a wide variety of assays have been used in previous studies, they have not been compared. Moreover, it is unclear whether some of the effects reported are specific for certain agents rather than reflecting the effects of FTIs in general. In the present study, several of these different approaches have been compared in tissue culture cell lines treated with two FTIs that have entered clinical testing, the nonpeptidomimetic FTI SCH66336 (31) and the peptidomimetic inhibitor FTI-277 (12). These experiments were designed to compare various assays of FT inhibition with respect to sensitivity and potential ease of application in the clinical setting and to determine whether these assays detect inhibition of FT by agents that are actually undergoing clinical testing.

MATERIALS AND METHODS

Materials and Methods. SCH66336 (32) was a kind gift from W. R. Bishop (Schering-Plough Research Institute, Kenilworth, NJ). FTI-277 (12) and mevastatin were purchased from Calbiochem (La Jolla, CA). [^{3}H]Mevalanolactone (specific activity, 50–60 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Plasmid pRc/CMV7SαpMEV encoding the mevalonic acid transporter was obtained from American Type Culture Collection (Manassas, VA).

Antibodies. Monoclonal antihuman lamin A (33) was a kind gift from Frank McKeon (Harvard Medical School, Boston, MA). A high-titer polyclonal serum that recognizes the COOH-terminal domain of human prelamin A was raised by immunizing rabbits with the peptide CLLGNSSPRTQSPQN coupled to keyhole limpet hemocyanin, as described by Sinensky *et al.* (26). Polyclonal rabbit anti-ras antiserum (antibody #3) was from Calbiochem (Cambridge, MA). Monoclonal anti-$p21^{waf1}$ (antibody #11) and anti-HDJ-2 were from Neomarkers (Fremont, CA). Polyclonal chicken sera that recognize lamins A and C or lamin B were raised as described previously (34). Affinity-purified peroxidase- and fluorochrome-coupled secondary antibodies were from Kirkegaard & Perry (Gaithersburg, MD).

Tissue Culture, Colony-forming Assays, and Immunoblotting. A549 human non-small cell lung cancer cells, HCT116 colon carcinoma cells, BxPC-3 pancreatic carcinoma cells, and MCF-7 breast cancer cells were obtained from American Type Culture Collection and propagated in the tissue culture media specified by the supplier. To assess the effect of SCH66336 on colony formation in A549 cells, aliquots containing 500 cells were plated in triplicate plates for each data point. After a 14–18 h incubation to allow cells to adhere, the indicated concentrations of SCH66336 (added from 1000-fold concentrated stocks prepared in DMSO) or the corresponding volume of diluent was added. Cells were incubated for 7–8 days to allow colonies to form. Alternatively, cells were incubated with SCH66336 for 24 h, washed twice in serum-free RPMI 1640, and incubated for 7 days in medium A (RPMI 1640, 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine) in the absence of SCH66336.

To prepare whole-cell lysates for immunoblotting, replicate 100-mm dishes containing 30–40% confluent cells were treated with the indicated concentrations of SCH66336 or diluent for 22–24 h. Alternatively, A549 cells were allowed to reach confluence and maintained in this state for 7 days prior to treatment with SCH66336 for 24 h. The highest concentration of SCH66336 used in these assays, 400 nM, is at or below the trough concentration achieved in patients on prolonged schedules of SCH66336 (35). Flow cytometry (36) confirmed that >95% of the cells were in G_0-G_1 after culture under confluent conditions. At the completion of the incubation, log phase or confluent cells were washed three times with ice-cold RPMI 1640 containing 10 mM HEPES (pH 7.4) and solubilized in buffer consisting of 6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C), 10 mM EDTA, 1% (v/v) β-mercaptoethanol, and 1 mM α-phenylmethylsulfonyl fluoride (freshly added from a 100 mM stock in anhydrous isopropanol). In some experiments, SCH66336 was replaced with 20 μM FTI-277 (added from a 1000-fold concentrated stock prepared in DMSO containing 10 mM DTT), a concentration chosen because it inhibited ras farnesylation and raf activation in tissue culture in a previous study (12).

After the whole-cell lysates were dialyzed and lyophilized as described previously (37), aliquots containing 50 μg of protein [assayed by the bicinchoninic acid method (38)] were subjected to electrophoresis on SDS-polyacrylamide gels containing 8% (w/v) acrylamide (for lamins or HDJ-2) or 12% acrylamide ($p21^{waf1}$, ras), transferred to nitrocellulose, and probed with the immunological reagents described above. Antigen-antibody complexes were detected using peroxidase-coupled secondary antibodies and ECL enhanced chemiluminescence reagents.

Transfection. A549 cells (2×10^7; 50% confluent) were transfected with 40 μg of plasmid pRc/CMV7αpMEV (prepared using a plasmid isolation kit; Qiagen, Valencia, CA) by electroporation using a Bio-Rad Gene Pulser electroporator operating at 300 V, 960 μfarad and infinite resistance. Cells were cultured at low density in medium A for 24 h. Stably transfected clones were selected in 2 mg/ml G418 and isolated using cloning rings. After expansion, clones were examined for ability to accumulate [^{3}H]mevalonate during a 22–24 h incubation at 37°C. Of 25 clones examined, one with substantial mevalonate uptake was identified and named A549pMEV.

Immunohistochemistry. A549 cells grown on 20-mm glass coverslips were incubated in medium A for a minimum of 1 week after reaching confluence. Cells were then treated with 200 nM SCH66336 or diluent for 24 h as described above. At the completion of the incubation, coverslips were washed twice with ice-cold PBS and fixed in acetone for 15 min at −20°C.

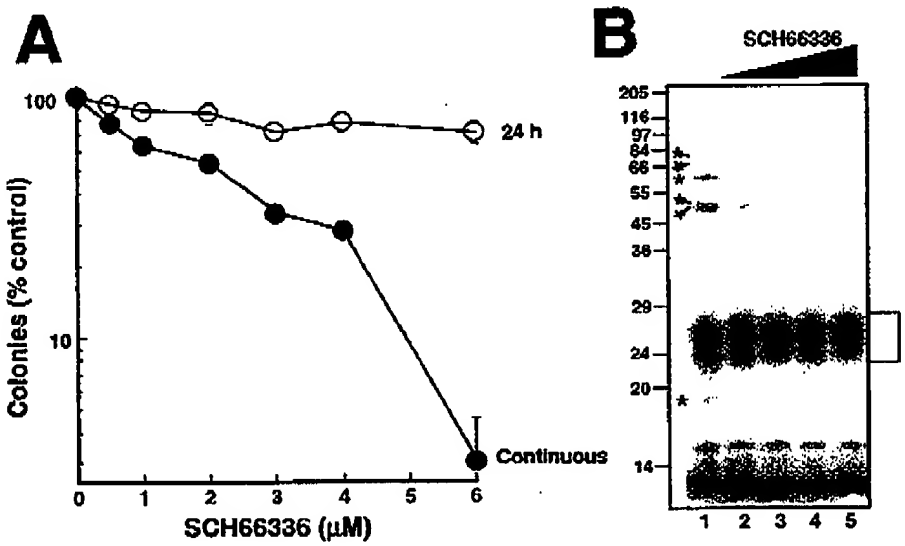

Fig. 1 Effect of SCH66336 on colony formation and protein prenylation in A549 cells. *A*, A549 cells were exposed to the indicated concentration of SCH66336 for 24 h, washed, and incubated in drug-free medium for 7 days (open circles). Alternatively, cells were exposed to SCH66336 continuously (●). *B*, A549pMEV cells were incubated for 24 h with 20 μM compactin and 1 μCi/ml [^{3}H]mevalonate in the presence of diluent (*Lane 1*) or SCH66336 at a concentration of 250 nM (*Lane 2*), 500 nM (*Lane 3*), 1000 nM (*Lane 4*), or 2000 nM (*Lane 5*). At the completion of the incubation, samples were subjected to SDS-PAGE and fluorography. *Numbers* at *left*, sizes of molecular weight markers (in thousands). *, species whose labeling decreases with SCH66336 treatment. *Bracket* at *right*, small GTP-binding proteins whose prenylation has been reported previously to be resistant to FTI treatment (11).

Samples were then rehydrated with two to three changes of PBS and blocked for a minimum of 1 h at 4°C in buffer B, which consisted of 10% (w/v) powdered milk in 150 mM NaCl, 10 mM Tris-HCl (pH 7.4 at 21°C), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 1 mM sodium azide. Cells were treated with a mixture of murine monoclonal anti-lamin A (1:3000) and rabbit anti-prelamin A (1:750) in buffer B at 4°C for 12–16 h. After removal of the primary antibodies, samples were washed six times with PBS over a 20-min period, incubated for 30 min with a mixture of affinity-purified, rhodamine-conjugated antimouse IgG and fluorescein-conjugated antirabbit IgG (20 μg/ml each), washed six times with PBS over a 20-min period, mounted in Vectashield (Vector Laboratories, Burlingame, CA), sealed with clear nail polish, and examined on a Zeiss LSM 310 confocal microscope (Carl Zeiss, Inc., New York, NY).

RESULTS

SCH66336 Inhibits Protein Prenylation in A549 Cells. In initial experiments, we examined the effects of the FTI SCH66336 (31, 32) on A549 cells, a non-small cell lung cancer line containing mutated K-ras (39, 40). Inclusion of increasing concentrations of SCH66336 in the tissue culture medium resulted in a dose-dependent decrease in the number of colonies that formed after 7–8 days (Fig. 1*A*, ●). In contrast, a 24-h exposure to SCH66336 had a minimal effect on colony formation (Fig. 1*A*, ○), indicating that SCH66336 was not killing the cells under these conditions. This model system provided the opportunity to examine the effects FT inhibition without secondary changes related to cytotoxic effects *per se*.

To confirm that SCH66336 was inhibiting protein farnesylation *in situ*, the covalent modification of various polypeptides by metabolic derivatives of mevalonic acid was examined in A549 cells transfected with the mevalonate transporter (Fig. 1*B*). This analysis revealed that SCH66336 caused a dose-dependent decrease in prenylation of several different polypeptides with apparent molecular weights between M_r ~18,000 and M_r ~75,000 (Fig. 1*B*, *). The prenylation of these polypeptides was detectably decreased at 250 nM SCH66336 (Fig. 1*B*, *Lane 2*) and completely inhibited at 1–2 μM (*Lanes 4* and *5*). These results confirm that SCH66336 inhibits the prenylation of several polypeptide species in this model system. In contrast, a number of polypeptides in the M_r 20,000–30,000 region were prenylated despite the presence of high concentrations of SCH66336 (Fig. 1*B*, *bracket*). These polypeptides, which were identified previously as small GTP-binding proteins, have been reported to be prenylated in the presence of other FTIs as well (11).

Examination of Altered Protein Mobility after FTI Treatment. In additional experiments, whole-cell lysates prepared from A549 cells after treatment with varying concentrations of SCH66336 were subjected to SDS-PAGE, followed by immunoblotting with reagents raised against polypeptides that are FT substrates. When blots were probed with a serum that recognizes all ras species, altered mobility of the predominant ras species in this cell line was not detectable (Fig. 2*A*, *top panel*). In the same samples, only a small percentage of the farnesylated nuclear protein lamin B (41) demonstrated altered mobility (Fig. 2*B*, *top panel*). In contrast, increased amounts of a slower migrating species of HDJ-2 were observed after

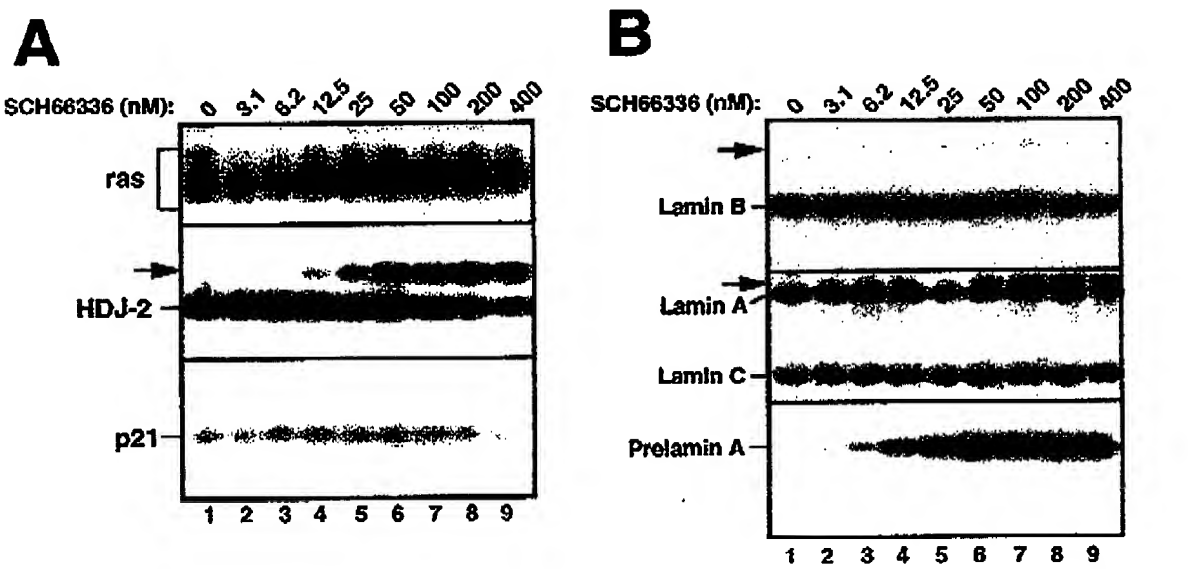

Fig. 2 Effect of SCH66336 on levels and migration of various polypeptides that have been proposed as markers of FT inhibition. *A*, A549 cells incubated for 24 h with the indicated concentration of SCH66336 were subjected to SDS-PAGE, followed by blotting with probes that recognize all ras isoforms (*top*), HDJ-2 (*middle*), or $p21^{waf1}$ (*bottom*). *B*, A549 cells treated for 24 h with the indicated concentration of SCH66336 were blotted with antisera that recognize lamin B (*top*), lamins A and C (*middle*), or the peptide LLGNSSPRTQSPQN at the COOH terminus of prelamin A (*bottom*). *Arrows*, slower migrating species of HDJ-2, lamin B, and lamin A in the respective panels.

SCH66336 treatment (Fig. 2*A*, *second panel*). This slower migrating species was readily detectable at drug concentrations as low as 6.25 nM and amounted to as much as half of the total HDJ-2 at 400 nM SCH66336.

When these same blots were probed with antibodies to the nuclear protein lamin A, a slower migrating species that was identified previously as prelamin A (23, 26) was seen at SCH66336 concentrations as low as 25 nM (Fig. 2*B*, *second panel*). Probing of the same blot with an antiserum that specifically recognizes the precursor polypeptide at the COOH terminus of prelamin A (26) revealed that the inhibition of prelamin A processing could be detected at SCH66336 concentrations as low as 6.25 nM (Fig. 2*B*, *bottom panel*).

To look for downstream markers that might be useful for examining FT inhibition, blots were also probed with antibodies to $p21^{waf1}$. Although another FTI has been reported to increase $p21^{waf1}$ levels in certain cells (27), levels of this polypeptide did not increase in A549 cells after treatment with SCH66336 (Fig. 2*A*, *bottom panel*).

To rule out the possibility that these results were unique to A549 cells, three additional cell lines were treated with 200 nM SCH66336 and subjected to the same analysis. The cell lines chosen for this analysis were the pancreatic carcinoma line BxPC-3, which has wild-type ras (42) and displays an IC_{50} of 200 nM upon continuous exposure to SCH66336 in clonogenic assays;[4] the breast carcinoma line MCF7, which has wild-type ras (43) and displays an IC_{50} of 50 nM upon continuous exposure to SCH66336 in clonogenic assays;[4] and the colon carcinoma line HCT116, which has a mutant K-ras (42) and displays an IC_{50} of 100 nM upon continuous exposure to SCH66336 in clonogenic assays.[4] Treatment for 24 h with 200 nM SCH66336 again failed to demonstrate any alteration in the mobility of detectable ras proteins by immunoblotting (Fig. 3, *top panel, Lane 2*). In contrast, increased levels of the slower migrating HDJ-2 species were readily detectable in all four cell lines after FTI treatment (Fig. 3, *second panel, Lane 2*). Moreover, each of the FTI-treated cell lines contained a slower migrating species of lamin A that reacted with the prelamin A antiserum (Fig. 3, *third panel, Lane 2*). As was the case in A549 cells, $p21^{waf1}$ failed to reproducibly increase in the other three cell lines after SCH66336 treatment (Fig. 3, *bottom panel, Lane 2*).

To rule out the possibility that the observed results were unique to the nonpeptidomimetic FTI SCH66336, all four cell lines were also treated with the peptidomimetic inhibitor FTI-277. Results of this analysis (Fig. 3, *Lanes 3*) demonstrated that FTI-277 altered processing of HDJ-2 and prelamin A. In contrast, altered migration of ras and altered levels of p21 were not observed. Identical results were also obtained when the FTI R115777 was substituted for SCH66336 (data not shown).

Effects of FT Inhibition on Noncycling Cells. Because the vast majority of cells in clinical tumor specimens are not cycling at any point in time, we evaluated these assays in noncycling A549 cells. Seven days after reaching confluence, the vast majority of these cells were in G_0-G_1 (Fig. 4*A*, *bottom panel*). In the confluence-arrested cells, increased levels of the slower migrating species of HDJ-2 and the appearance of pre-

[4] J. N. Davis and A. A. Adjei, unpublished observations.

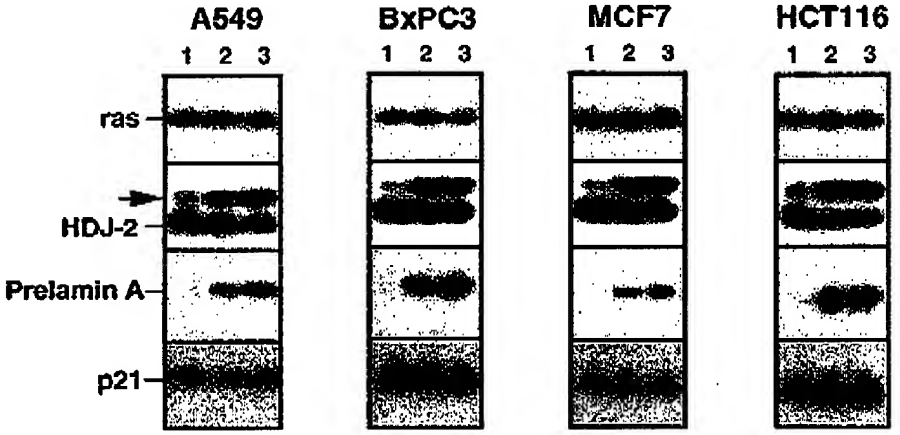

Fig. 3 Evaluation of assays in various cell types. A549, HCT116, BxPC-3, and MCF-7 cells were treated for 24 h with diluent (*Lane 1*), 200 nM SCH66336 (*Lane 2*), or 20 μM FTI-277 (*Lane 3*). At the completion of the incubation, whole-cell lysates were subjected to SDS-PAGE, followed by transfer to nitrocellulose and blotting with reagents that recognize all ras isoforms (*top panel*), HDJ-2 (*second panel*), prelamin A (*third panel*), or $p21^{waf1}$ (*bottom panel*). Note that both drugs induced increases in the slower migrating species of HDJ-2 and prelamin A in all four cell lines.

lamin A were readily demonstrated after SCH66336 treatment (Fig. 4*A*), although the signals were slightly decreased in noncycling cells compared with cycling A549 cells examined on the same blot.

Immunohistochemical Staining for Prelamin A. Of the markers examined above, prelamin A is unique in containing a large peptide that is absent from the processed protein. It has been suggested previously that the presence of prelamin A can be detected by immunohistochemistry after treatment of log phase cells with the benzodiazepine-based FTI BZA-5*B* (26). To investigate the potential usefulness of this assay with other FTIs, as well as determine the suitability of this approach for assessing FTI action in noncycling cells, confluence-arrested A549 cells were treated with SCH66336 and then stained for prelamin A in the absence or presence of the peptide used to raise the serum. Results of this analysis revealed that prelamin A was at the limit of detection in untreated cells (Fig. 4*B*, *top row*) but became readily detectable in all of the cells by immunofluorescence after treatment with SCH66336 (Fig. 4*B*, *middle row*). The immunizing peptide prevented labeling with this serum (Fig. 4*B*, *bottom row*), demonstrating specificity of this reaction. When combined with the immunoblotting results in Fig. 3, these observations not only confirm that multiple FTIs inhibit prelamin A processing but also indicate that this inhibition can be detected in noncycling cells by immunoblotting (Fig. 4*A*) or immunohistochemistry (Fig. 4*B*).

DISCUSSION

The present study compared the performance of several different assays that have been proposed for monitoring FT inhibition in the clinical setting. This study used the nonpeptidomimetic FTI SCH66336 and the peptidomimetic inhibitor FTI-277, both of which have entered clinical trials. Results of these assays were compared in four separate cell lines and in log-phase *versus* confluent, noncycling cells. These results have potential implications for monitoring FT inhibition in the clinical setting.

Because the FTIs are among the first inhibitors of signal transduction to be widely tested in the clinic, there is considerable interest in assessing their effects in clinical material. An ideal marker for such studies would be one that is highly sensitive to inhibition of the targeted pathway, selective for inhibition of that pathway, and assayable in a wide variety of tumor types as well as potentially in surrogate normal tissues. Other features that would be desirable in a marker assay would be ease of performance and the ability to analyze samples in batches rather than individually. As signal transduction inhibitors are tested clinically, such assays will be particularly important in differentiating between compounds that successfully target the intended pathway but do not have clinical activity *versus* compounds that fail to target the intended pathway at concentrations where toxicity prohibits dose escalation. Distinguishing between these alternatives will be particularly important in clinical trials that do not have the classical end point of tumor shrinkage as well as in chemoprevention and adjuvant chemotherapy trials. In light of the potential importance of these types of studies, we have compared several of the assays that have been proposed for the assessment of FT inhibition in clinical material.

One potential approach would be to assay FT enzymatic activity in cell extracts after drug exposure. This approach suffers from the potential problem that reversible inhibitors such as SCH66336 and FTI-277 might dissociate from FT during cell purification and fractionation, thereby leading to underestimation of the degree of inhibition achieved *in situ*. In addition, current assays of FT activity in certain cell types are subject to interference by endogenous inhibitors.[5] As a result, some investigators have resorted to partial purification of the enzyme prior to assaying its activity (44), an approach that does not appear feasible in the context of multicenter clinical trials.

Immunoblotting and immunohistochemistry approaches appear to be better suited for application in the clinical setting. FTIs were initially developed to inhibit the posttranslational processing of the ras oncoproteins (4, 5, 45). Several earlier

[5] D. End, personal communication.

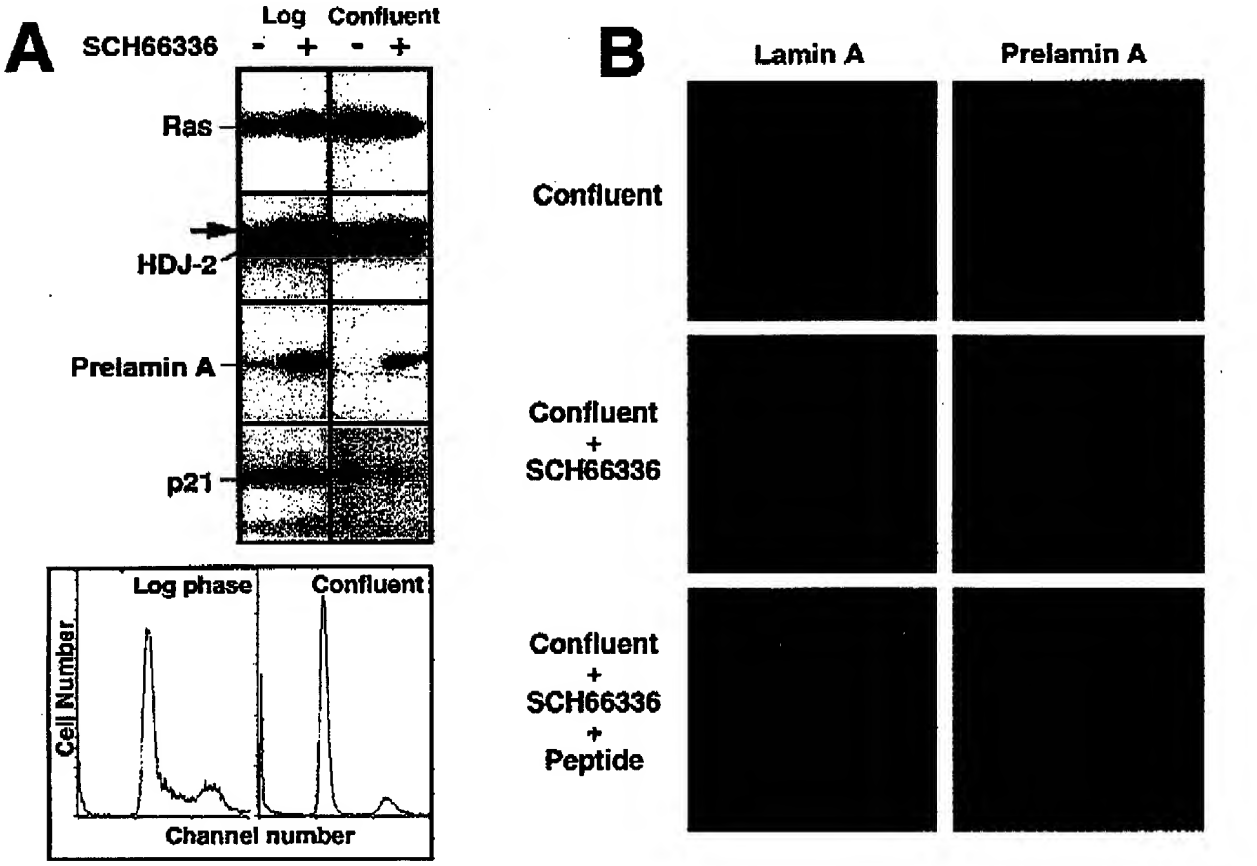

Fig. 4 Changes in HDJ-2 and prelamin A in noncycling cells. *A*, log phase or confluence-arrested A549 cells were treated with diluent or 200 nM SCH66336 for 24 h as indicated. Whole-cell lysates were subjected to SDS-PAGE, followed by immunoblotting with the indicated probe. *Bottom*, DNA histograms of log phase and confluence-arrested A549 cells. *B*, evaluation of prelamin A processing in noncycling cells. One week after reaching confluence, A549 cells were incubated for 24 h with diluent (*top row*) or 200 nM SCH66336 (*middle* and *bottom rows*). After the cells were fixed in acetone, double label indirect immunofluorescence was performed using monoclonal anti-lamin A (*left*) and polyclonal antiserum that recognizes the precursor peptide at the COOH terminus of lamin A (*right*). The incubation with primary antibodies was performed in the absence (*top two rows*) or presence (*bottom row*) of 10 μg/ml CLLGNSSPRTQSPQN, the peptide that was used to raise the prelamin A-specific serum. In each row, the same field was imaged using excitation wavelengths of 568 nm (*left*) or 488 nm (*right*).

studies demonstrated that FTI treatment altered mobility of H-ras in transfected murine cells. Nonetheless, immunoblotting experiments failed to demonstrate altered migration of endogenous ras polypeptides in a number of different cell lines after treatment with SCH66336 or FTI-277 (Figs. 2 and 3). There are several potential explanations for this result. It is possible that the predominant ras species in these cell lines are isoforms of K-ras, which has been shown to be more resistant to FTIs than H-ras (46). We note, however, that prelamin A farnesylation is also more resistant to FTIs than H-ras (47), yet we could readily detect inhibition of prelamin A processing in each of the cell lines. Alternatively, it is possible that geranylgeranylation of ras polypeptides in FTI-treated human cells (16) leads to processed ras species that have unaltered mobility on SDS-polyacrylamide gels. In either case, it is possible that immunoprecipitation of H-ras, followed by Western blotting (an approach necessitated by low levels of endogenous H-ras in these cells and many other tissue types), might demonstrate an effect of the FTIs on migration of H-ras. However, it is difficult to envision that immunoprecipitation followed by immunoblotting would be practical for routine performance in the clinical setting.

Recent studies have suggested that RhoB might also be a critical target for FTIs (4, 10, 17–19). These studies have examined epitope-tagged RhoB that is expressed after transfection. In our hands, experiments similar to those in Fig. 2 have revealed that endogenous rho species are below the limit of detection with commercially available monoclonal antibodies,[4] making it difficult to use this polypeptide as a marker of FTI action in the clinical setting. Accordingly, we focused on other potential markers of FT inhibition.

Consistent with recent results published in abstract form (24), the prenylated protein HDJ-2 demonstrated a shift in

mobility upon treatment with SCH66336 (Figs. 2 and 3). The appearance of the slower migrating species was detectable at concentrations of SCH66336 as low as 6.25 nM (Fig. 2*A*). In addition, a similar mobility shift was observed in noncycling cells (Fig. 4*A*), indicating the potential usefulness of this assay in clinical tumor specimens.

Work from several laboratories demonstrated previously that lamin B and prelamin A are farnesylated polypeptides (23, 41). In our studies, only a small amount of lamin B displayed a mobility shift in the presence of SCH66336 (Fig. 2*B*). In contrast, as much as half of the lamin A demonstrated a mobility shift after the same treatment (Fig. 2*B*). Further analysis revealed that the processing of prelamin A was extensively inhibited in all cell lines examined (Figs. 2*B* and 3). Accumulation of prelamin A above low basal levels was detectable at SCH66336 concentrations as low as 6.25 nM and increased in a dose-dependent manner (Fig. 2*B*). Because prelamin A is ordinarily present in limited amounts within the cell, the assay for prelamin A was suitable for immunoblotting (Figs. 2–4) or immunohistochemistry (Fig. 4*B*), either of which should be potentially useful in the clinical setting. The widespread use of immunohistochemistry for other purposes (*e.g.*, assessment of estrogen and progesterone receptors, HER2-neu status, and cytokeratin expression) suggests that the immunohistochemical assay for prelamin A might be particularly useful.

Finally, as a potential example of polypeptide changes downstream from ras, we examined the up-regulation of $p21^{waf1}$. Although the FTI L744832 has been reported to induce accumulation of $p21^{waf1}$ in a p53-dependent manner, we could not detect increased $p21^{waf1}$ in any of the four cell lines after treatment with SCH66336 (Figs. 2 and 3) or FTI-277 (Fig. 3). In this context, it is important to note that A549, MCF-7, and HCT116 cells all have wild-type p53. These observations not only raise the possibility that up-regulation of $p21^{waf1}$ after treatment with L744832 might be a drug- or cell line-specific effect but also highlight the importance of examining potential markers of FTI action with the compounds that are actually undergoing clinical testing.

To rule out the possibility that some of the results described in the present work might be unique to SCH66336 and FTI-277, experiments depicted in Figs. 2–4 were repeated using R115777, another FTI that is in Phase II clinical trials (10). All of the results presented above were readily reproduced with R115777.[4]

In summary, the current findings identify two different assays that appear to be sensitive and widely applicable markers of FT inhibition. HDJ-2 is present in a variety of tumor cell lines and undergoes a readily detectable mobility shift upon treatment with FTIs. Lamin A likewise undergoes a mobility shift in multiple cell lines after FTI treatment. The mobility shift of HDJ-2 and the accumulation of prelamin A are detectable at similar drug concentrations. The retention of a unique prepeptide in prelamin A after FTI treatment makes this polypeptide amenable to assay by both immunoblotting and immunohistochemistry. Because the effects on HDJ-2 and lamin A are observed in noncycling as well as cycling cells, one or both of these assays should be suitable for detecting inhibition of FT in the clinical setting.

ACKNOWLEDGMENTS

We gratefully acknowledge kind gifts of SCH66336 from Schering-Plough Research Institute and R115777 from the Janssen Research Foundation as well as antibodies from Michael Sinensky and Frank McKeon. We also thank Ed Sausville, Susan Arbuck, Louise Grochow, David Cutler, W. Robert Bishop, and Paul Kirschmeier for provocative discussions and Deb Strauss for secretarial assistance.

REFERENCES

1. Clarke, S. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Annu. Rev. Biochem., *61:* 355–386, 1992.

2. Sinensky, M., and Lutz, R. J. The prenylation of proteins. Bioessays, *14:* 25–31, 1992.

3. Bos, J. L. *ras* oncogenes in human cancer. A review. Cancer Res., *49:* 4682–4689, 1989.

4. Cox, A. D., and Der, C. J. Farnesyltransferase inhibitors and cancer treatment: targeting simply ras? Biochim. Biophys. Acta, *1333:* F51–F71, 1997.

5. Gibbs, J. B., and Oliff, A. The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. Annu. Rev. Pharmacol. Toxicol., *37:* 143–166, 1997.

6. Gelb, M. H., Scholten, J. D., and Sebolt-Leopold, J. S. Protein prenylation: from discovery to prospects for cancer treatment. Curr. Opin. Chem. Biol., *2:* 40–48, 1998.

7. Beaupre, D. M., and Kurzrock, R. RAS and leukemia: from basic mechanisms to gene-directed therapy. J. Clin. Oncol., *17:* 1071–1079, 1999.

8. Rowinsky, E. K., Windle, J. J., and Von Hoff, D. D. Ras protein farnesyltransferase. A strategic target for anticancer therapeutic development. J. Clin. Oncol., *17:* 3631–3652, 1999.

9. Khosravi-Far, R., Cox, A. D., Kato, K., and Der, C. J. Protein prenylation: key to ras function and cancer intervention? Cell Growth Differ., *3:* 461–469, 1992.

10. End, D. W. Farnesyl protein transferase inhibitors and other therapies targeting the ras signal transduction pathway. Investig. New Drugs, *17:* 241–258, 1999.

11. James, G. L., Goldstein, J. L., Brown, M. S., Rawson, T. E., Somers, T. C., McDowell, R. S., Crowley, C. W., Lucas, B. K., Levinson, A. D., and Marsters, J. C. Benzodiazepine peptidomimetics: potent inhibitors of ras farnesylation in animal cells. Science (Washington DC), *260:* 1937–1942, 1993.

12. Lerner, E. C., Qian, Y., Blaskovich, M. A., Fossum, R. D., Vogt, A., Sun, J., Cox, A. D., Der, C. J., Hamilton, A. D., and Sebti, S. M. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic *ras* signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. J. Biol. Chem., *270:* 26802–26806, 1995.

13. Kohl, N. E., Wilson, F. R., Thomas, T. J., Bock, R. L., Mosser, S. D., Oliff, A., and Gibbs, J. B. Inhibition of ras function *in vitro* and *in vivo* using inhibitors of farnesyl-protein transferase. Methods Enzymol., *255:* 378–386, 1995.

14. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. Biological assays for ras transformation. Methods Enzymol., *255:* 395–412, 1995.

15. Osman, H., Mazet, J. L., Maume, G., and Maume, B. F. Geranylgeranyl as well as farnesyl moiety is transferred to p21 overproduced in adrenocortical cells transformed with c-Ha-rasEJ oncogene. Biochem. Biophys. Res. Commun., *231:* 789–792, 1997.

16. Whyte, D. B., Kirschmeier, P., Hockenberry, T. N., Nunez-Oliva, I., James, L., Catino, J. J., Bishop, W. R., and Pai, J. K. K- and N-ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J. Biol. Chem., *272:* 14459–14464, 1997.

17. Gibbs, J. B., Graham, S. L., Hartman, G. D., Koblan, K. S., Kohl, N. E., Omer, C. A., and Oliff, A. Farnesyltransferase inhibitors *versus* ras inhibitors. Curr. Opin. Chem. Biol., *1:* 197–203, 1997.

18. Lebowitz, P. F., and Prendergast, G. C. Non-ras targets of farnesyltransferase inhibitors. Focus on Rho. Oncogene, *17:* 1439–1445, 1998.

19. Du, W., Lebowitz, P. F., and Prendergast, G. C. Cell growth inhibition by farnesyltransferase inhibitors is mediated by gain of geranylgeranylated Rho B. Mol. Cell. Biol., *19:* 1831–1840, 1999.

20. Neckers, L., Mimnaugh, E., and Schulte, T. W. The Hsp90 chaperone family. *In:* D. S. Latchman (ed.), Stress Proteins, pp. 9–42. New York: Springer-Verlag, 1999.

21. James, G. L., Goldstein, J. L., Pathak, R. K., Anderson, R. G., and Brown, M. S. PxF, a prenylated protein of peroxisomes. J. Biol. Chem., *269:* 14182–14190, 1994.

22. McKeon, F. D., Kirschner, M. W., and Caput, D. Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. Nature (Lond.), *319:* 463–468, 1986.

23. Beck, L. A., Hosick, T. J., and Sinensky, M. Isoprenylation is required for the processing of the lamin A precursor. J. Cell Biol., *110:* 1489–1499, 1990.

24. Britten, C. D., Rowinsky, E., Yao, S-L., Soignet, S., Rosen, N., Eckhardt, S. G., Drengler, R., Hammond, L., Sui, L. L., Smith, L., McCreery, H., Pezzulli, S., Lee, Y., Lobell, R., Deutsch, P., Von Hoff, D., and Spriggs, D. The farnesyl protein transferase (FPTase) inhibitor L-778,123 in patients with solid cancers. Proc. Am. Soc. Clin. Oncol., *18:* 155A, 1999.

25. Kilic, F., Dalton, M. B., Burrell, S. K., Mayer, J. P., Patterson, S. D., and Sinensky, M. *In vitro* assay and characterization of the farnesylation-dependent prelamin A endoprotease. J. Biol. Chem., *272:* 5298–5304, 1997.

26. Sinensky, M., Fantle, K., and Dalton, M. An antibody which specifically recognizes prelamin A but not mature lamin A: application to detection of blocks in farnesylation-dependent protein processing. Cancer Res., *54:* 3229–3232, 1994.

27. Sepp-Lorenzino, L., and Rosen, N. A farnesyl-protein transferase inhibitor induces p21 expression, and G_1 block in p53 wild type tumor cells. J. Biol. Chem., *273:* 20243–20251, 1998.

28. Stokoe, D., MacDonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. Activation of Raf as a result of recruitment to the plasma membrane. Science (Washington DC), *264:* 1463–1467, 1994.

29. Khosravi-Far, R., Campbell, S., Rossman, K. L., and Der, C. J. Increasing Complexity of ras signal transduction: involvement of Rho family proteins. Adv. Cancer, *72:* 57–107, 1998.

30. Plattner, R., Gupta, S., Khosravi-Far, R., Sato, K. Y., Perucho, M., Der, C. J., and Stanbridge, E. J. Differential contribution of the ERK and JNK mitogen-activated protein kinase cascades to ras transformation of HT1080 fibrosarcoma and DLD-1 colon carcinoma cells. Oncogene, *18:* 1807–1817, 1999.

31. Njoroge, F. G., Taveras, A. G., Kelly, J., Temiszewski, S., Mallams, A. K., Wolin, R., Afonso, A., Cooper, A. B., Rane, D. F., Liu, Y. T., Wong, J., Vibulbhan, B., Pinto, P., Deskus, J., Alvarez, C. S., del Rosario, J., Connolly, M., Wang, J., Desai, J., Rossman, R. R., Bishop, W. R., Patton, R., Wang, L., Kirschmeier, P., and Ganguly, A. K. (+)-4-[2-[4-(8-Chloro-3,10-dibromo-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-b]-pyridin-11(*R*)-yl)-1-piperidinyl]-2-oxo-ethyl]-1-piperidinecarboxamide (SCH-66336). A very potent farnesyl protein transferase inhibitor as a novel antitumor agent. J. Med. Chem., *41:* 4890–4902, 1998.

32. Liu, M., Bryant, M. S., Chen, J., Lee, S., Yaremko, B., Lipari, P., Malkowski, M., Ferrari, E., Nielsen, L., Prioli, N., Dell, J., Sinha, D., Syed, J., Korfmacher, W. A., Nomeir, A. A., Lin, C. C., Wang, L., Taveras, A. G., Doll, R. J., Njoroge, F. G., Mallams, A. K., Remiszewski, S., Catino, J. J., Girijavallabhan, V. M., and Bishop, W. R. Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and Wap-Ras transgenic mice. Cancer Res., *58:* 4947–4956, 1998.

33. Loewinger, L., and McKeon, F. Mutations in the nuclear lamin proteins resulting in their aberrant assembly in the cytoplasm. EMBO J., *7:* 2301–2309, 1988.

34. Kaufmann, S. H. Additional members of the rat liver lamin polypeptide family: structural and immunological characterization. J. Biol. Chem., *264:* 13946–13955, 1989.

35. Eskens, F., Awada, A., Verweij, J., Cutler, D. L., Hanauske, A., and Piccart, M. Phase I and pharmacologic study of continuous daily oral SCH66336, a novel farnesyl transferase inhibitor, in patients with solid tumors. Proc. Am. Soc. Clin. Oncol., *18:* 600, 1999.

36. Bible, K., and Kaufmann, S. H. Cytotoxic synergy between flavopiridol (NSC 649890, L86–8275) and various antineoplastic agents: importance of sequence of administration. Cancer Res., *57:* 3375–3380, 1997.

37. Kaufmann, S. H., Svingen, P. A., Gore, S. D., Armstrong, D. K., Cheng, Y-C., and Rowinsky, E. K. Altered formation of topotecan-stabilized topoisomerase I-DNA adducts in human leukemia cells. Blood, *89:* 2098–2104, 1997.

38. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. Measurement of protein using bicinchoninic acid. Anal. Biochem., *150:* 76–85, 1985.

39. Mitchell, C. E., Belinsky, S. A., and Lechner, J. F. Detection and quantitation of mutant K-ras codon 12 restriction fragments by capillary electrophoresis. Anal. Biochem., *224.* 148–153, 1995.

40. Valenzuela, D. M., and Groffen, J. Four human carcinoma cell lines with novel mutations in position 12 of c-K-*ras* Oncogene. Nucleic Acids Res., *14:* 843–852, 1986.

41. Farnsworth, C. C., Wolda, S. L., Gelb, M. H., and Glomset, J. A. Human lamin B contains a farnesylated cysteine residue. J. Biol. Chem., *264:* 20422–20429, 1989.

42. Rajesh, D., Schell, K., and Verma, A. K. Ras mutation, irrespective of cell type and p53 status, determines a cell's destiny to undergo apoptosis by okadaic acid, an inhibitor of protein phosphatase 1 and 2A. Mol. Pharmacol., *56:* 515–525, 1999.

43. Gelmann, E. P., Thompson, E. W., and Sommers, C. L. Invasive and metastatic properties of MCF-7 cells and rasH-transfected MCF-7 cell lines. Int. J. Cancer, *50:* 665–669, 1992.

44. James, G. L., Brown, M. S., and Goldstein, J. L. Assays for inhibitors of CAAX farnesyltransferase in *vitro* and in intact cells. Methods Enzymol., *255:* 38–46, 1995.

45. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. Isoprenoid addition to Ras proteins is the critical modification for its membrane association and transforming activity. Proc. Natl. Acad. Sci., USA, *89:* 6403–6407, 1992.

46. Sepp-Lorenzino, L., Ma, Z., Rands, E., Kohl, N. E., Gibbs, J. B., Oliff, A., and Rosen, N. A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. Cancer Res., *55:* 5302–5309, 1995.

47. Garcia, A. M., Rowell, C., Ackermann, K., Kowalczyk, J. J., and Lewis, M. D. Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. J. Biol. Chem., *268:* 18415–18418, 1993.

3544 Vol. 7, 3544–3550, November 2001 Clinical Cancer Research

Preclinical Antitumor Activity and Pharmacodynamic Studies with the Farnesyl Protein Transferase Inhibitor R115777 in Human Breast Cancer

Lloyd R. Kelland,[1] Vicki Smith, Melanie Valenti, Lisa Patterson, Paul A. Clarke, Simone Detre, Dave End, Angela J. Howes, Mitch Dowsett, Paul Workman, and Stephen R. D. Johnston

CRC Centre for Cancer Therapeutics, The Institute of Cancer Research, Surrey SM2 5NG, United Kingdom [L. R. K., V. S., M. V., L. P., P. A. C., P. W.]; Departments of Biochemistry [S. D., M. D.] and Medicine [S. R. D. J.], Royal Marsden Hospital, London SW3 6JJ, United Kingdom; Janssen Research Foundation, Spring House, Pennsylvania 19477 [D. E.]; and Janssen-Cilag Ltd, High Wycombe, Buckinghamshire HP14 4HJ, United Kingdom [A. J. H.]

ABSTRACT

Antitumor and pharmacodynamic studies were performed in MCF-7 human breast cancer cells and companion xenografts with the farnesyl protein transferase inhibitor, R115777, presently undergoing Phase II clinical trials, including in breast cancer. R115777 inhibited growth of MCF-7 cells *in vitro* with an IC_{50} of 0.31 ± 0.25 μM. Exposure of MCF-7 cells to increasing concentrations of R115777 for 24 h resulted in the inhibition of protein farnesylation, as indicated by the appearance of prelamin A at concentrations >1 μM. After continuous exposure to 2 μM R115777, prelamin A levels peaked at 2 h post drug exposure and remained high for up to 72 h. R115777 administered p.o. twice daily for 10 consecutive days to mice bearing established s.c. MCF-7 xenografts induced tumor inhibition at a dose of 25 mg/kg [percentage of treated *versus* control (% T/C) = 63% at day 21]. Greater inhibition was observed at doses of 50 mg/kg (% T/C at day 21 = 38%) or 100 mg/kg (% T/C at day 21 = 43%). The antitumor effect appeared to be mainly cytostatic with little evidence of tumor shrinkage to less than the starting volume. Tumor response correlated with an increase in the appearance of prelamin A, but no changes in the prenylation of lamin B, heat shock protein 40, or N-Ras were detectable. In addition, significant increases in apoptotic index and $p21^{WAF1/CIP1}$ expression were observed, concomitant with a decrease in proliferation as measured by Ki-67 staining. An increase in prelamin A was also observed in peripheral blood lymphocytes in a breast cancer patient who responded to R115777. These data show that R115777 possesses preclinical antitumor activity against human breast cancer and that the appearance of prelamin A may provide a sensitive and convenient pharmacodynamic marker of inhibition of prenylation and/or response.

INTRODUCTION

Approximately 10 years ago it was discovered that Ras oncoproteins require localization to the inner surface of the cell membrane to exert their cellular mitogenic activity (1, 2). The first and most important step in this process is a post-translational modification in which a C15 farnesyl group is transferred from farnesyl diphosphate to the sulfur atom of the cysteine residue of the COOH-terminal tetrapeptide CAAX of Ras through the activity of the enzyme, FPT.[2] Consequently, many attempts have been made to design and synthesize inhibitors of FPT.

Following the demonstration of inhibition of *ras*-dependent transformation of fibroblasts (3) and *in vivo* antitumor activity against carcinomas in *ras* transgenic mice (4), at least four FTIs recently entered clinical trials: SCH66336 (5); R115777 (6, 7); L-778,123; and BMS-214662 (8–10). Among these is the imidazole-based compound, R115777, which is a competitive inhibitor of the CAAX peptide binding site of FPT with a K_i of 0.5 nM and which inhibits the farnesylation of lamin B1 (IC_{50} = 0.8 nM) and K-ras (IC_{50} = 7.9 nM; Ref. 11). Furthermore, R115777 inhibited the proliferation of both H-ras-transformed fibroblasts (IC_{50} = 1.7 nM) and human colon and pancreatic cell lines possessing K-*ras* mutations (IC_{50} = 16–22 nM) and showed potent oral antitumor activity against human LoVo colon and human CAPAN-2 pancreatic xenografts (12–14). An initial Phase I trial with oral R115777 (25–1300 mg twice daily for 5 days every 2 weeks) identified dose-limiting toxicities of neuropathy and fatigue with some nausea, vomiting, and headache (6). Additional trials using either continuous twice-daily oral dosing or dosing for 21 days followed by 7 days of rest, reported a dose-limiting toxicity of myelosuppression (7). The recommended dose for Phase II trials based on this schedule was 300 mg p.o. twice daily.

Although the incidence of *ras* mutations in breast cancer is relatively low (<5%; Ref. 15), aberrant function of the Ras signal transduction pathway is common (*e.g.*, through upstream activation via HER2 or epidermal growth factor receptor; Ref.

Received 3/30/01; revised 7/13/01; accepted 8/7/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at CRC Centre for Cancer Therapeutics, The Institute of Cancer Research, 15 Cotswold Road, Surrey SM2 5NG, United Kingdom. Phone: (44) 20 8722 4261; Fax: (44) 20 8722 4101; E-mail: lloyd@icr.ac.uk.

[2] The abbreviations used are: FPT, farnesyl protein transferase; FTI, FPT inhibitor; PI3, phosphatidylinositol 3′-kinase; PBL, peripheral blood lymphocyte; HSP, heat shock protein; SRB, sulforhodamine B; T/C, treated *versus* control; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling.

15). Furthermore, in recent years it has become apparent that the FTIs, although originally envisaged as Ras inhibitors, may confer their antitumor properties through intracellular effects on additional proteins that require farnesylation. These include lamins A and B, Rap2, Rho B and E, and the centromere-associated proteins CENPE and CENPF (8, 10, 16). Although compelling data suggest that specific substrates such as Rho B may be the key determinant of sensitivity to FTIs (17), other data are indicative of effects on alternative proteins/pathways to the K-Ras/Raf/MEK/Erk pathway, such as the H-Ras/PI3 kinase/AKT pathway (18–20). In addition to farnesylation, some proteins are post-translationally modified by the addition of a 20-carbon geranylgeranyl moiety via the enzyme geranylgeranyl protein transferase I (21, 22). It has been reported that K-Ras may become geranylgeranylated and maintain transforming activity when FPT is inhibited in some cell lines (21, 22). These findings have led to particular challenges in the choice and design of Phase II trials with these agents and in the rational development of pharmacodynamic end points of response or toxicity.

Among various Phase II clinical trials of R115777, we have conducted a trial in women with advanced breast cancer (23). In conjunction with this trial, the aims of this preclinical study were to assess the activity of R115777 in a cell line and a companion xenograft model of human breast cancer and to investigate possible pharmacodynamic markers of response that could then be applied to PBLs or tumor biopsy specimens. On the basis of a recent report (24), particular emphasis was placed on monitoring of the appearance of unprocessed prelamin A (25) as a pharmacodynamic readout of response, although other proteins known to be prenylated (N-Ras, lamin B, and HSP40) were also investigated.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line MCF-7 (and the A2780 human ovarian carcinoma and HT29 colon carcinoma cell lines) was grown as a monolayer in DMEM containing 10% heat-inactivated FCS, 2 mM L-glutamine, and minimal nonessential amino acids (Life Technologies, Inc., Paisley, Scotland) in a 6% CO_2-94% air atmosphere.

Cell Growth Inhibition. The sensitivity of human tumor cell lines, including MCF-7, was assessed by 96-h drug exposure. R115777 was dissolved in acidified water at 1 mM and diluted in growth medium immediately prior to its addition to cells seeded overnight in 96-well microtiter plates. Quantitation of cell numbers in treated (four replicate wells) *versus* control (four replicates) wells was assessed by SRB staining as described previously (26). Drug potency was determined in terms of IC_{50}s.

Human Tumor Xenografts. The MCF-7 line was grown as s.c. xenografts in female athymic nude mice (nu/nu) by passage of 2-mm diameter pieces of tumor. Growth was maintained by estrogen supplementation through intradermal injection of estrogen pellets (dose, 1.7 mg over 60 days; Innovative Research of America, Sarasota, FL). When tumors had reached a largest diameter of ~7 mm, mice were randomized to receive either drug vehicle [20% (w/v) β-cyclodextrin (pH 2.5)] or R115777 administered by oral gavage at doses of 25, 50, and 100 mg/kg. Dosing was twice daily for 10 consecutive days. There were 11 tumor-bearing animals in each group; 6 were used for the therapy arm, and 5 were harvested on day 11 (1 day after the final dose) for pharmacodynamic measurements. For the therapy arm, tumor growth was assessed twice weekly in control and treated groups by caliper measurements of the two largest diameters. Volumes were then calculated according to the formula: volume $= a \times b^2 \times \pi/6$, where a and b are orthogonal tumor diameters. Tumor volumes were then expressed as a percentage of the volume at the start of treatment (relative tumor volume). The effect of the drug was determined by the growth delay (difference in time, in days, for the volumes of control *versus* treated tumors to double) or % T/C on day 21, as used previously (27).

All animal procedures were performed within local and national ethics guidelines.

Western Blotting. Immunoblotting of proteins was carried out as described previously (*e.g.*, Ref. 28), using asynchronized cells in exponential growth. In addition, assessments were made using xenograft material harvested on day 11 (24 h after the final dose of drug) and PBLs taken from patients participating in the Phase II R115777 breast cancer trial (pretreatment and after 4 weeks of receiving 300 mg twice daily every day). PBLs were prepared using Lymphoprep (Nycomed) and centrifugation at 800 × *g* for 20 min at room temperature. PBLs were then separated, washed in PBS by centrifugation, pelleted, and snap frozen in liquid nitrogen. Xenograft samples were frozen in liquid nitrogen and homogenized, and protein was extracted. Proteins (typically 50 μg/lane) were separated by SDS-PAGE and electroblotted to nitrocellulose filters. A polyclonal antibody to the COOH-terminal domain of human prelamin A (24) was kindly provided by Drs. A. Adjei and S. Kaufmann (Mayo Clinic, Rochester, MN). Antibodies to lamin B (Santa Cruz Biotechnology C-20), N-Ras (Santa Cruz Biotechnology C-20), and HSP40 (Santa Cruz Biotechnology N-19) were used at dilutions of 1:1000, 1:1000, and 1:500, respectively. Secondary antibodies were purchased from Amersham Pharmacia.

Measurements of Proliferation and Apoptosis. Markers for proliferation and apoptosis were assessed by immunohistochemistry of formalin-fixed xenograft tissue (taken on day 11), as described previously, from MCF-7 xenografts treated with drug vehicle or with R115777 as above (29, 30). Sections (3 μM) were cut from treated and control formalin-fixed, paraffin wax-embedded xenograft tissue and placed on charged slides. Proliferation was analyzed in sections after they had been dewaxed in xylene and gradually rehydrated with water, and endogenous peroxidase activity was blocked. Antigen retrieval was performed by microwaving sections at 750 W in citrate buffer (pH 6.0) for 10 min, after which buffered sections were cooled to room temperature and a blocking antibody was applied in PBS (pH 7.4) prior to addition of the primary antibody. The sections were incubated for 45 min in 1:200 biotinylated antimouse immunoglobulins and rinsed; streptavidin ABC-horseradish peroxidase complex (Dako) was then applied for 30 min. After sections were washed in PBS, the peroxidase reaction was developed to a brown stain by the addition of 0.05% diaminobenzidine enhanced with 0.07% imidazole and hydrogen peroxide. Cell cytoplasm was counterstained blue with

Mayer's hematoxylin, and sections were dehydrated, cleared in xylene, and permanently mounted in DePex.

Proliferation was assessed by Ki-67 immunostaining using the MIB1 antibody (The Binding Site Ltd., Birmingham, United Kingdom) at a dilution of 1:50 for 1 h. Ki-67 cells positive for nuclear staining were recorded as percentages. Sections were stained in one batch together with known positive controls and scored by one individual. Ki-67 cells positive for nuclear staining were recorded as percentages. The method for immunocytochemical detection of p21 protein was the same as described above for Ki-67, except that WAF1 (Ab-1) primary monoclonal antibody (Oncogene Research Products, Calbiochem) was used at a dilution of 1:30 for 1 h. Cells with positive nuclear staining for p21 were assessed as percentages.

Apoptosis was determined by the TUNEL assay as described previously, using xenograft tumor sections (31). All sections were examined under a standard light microscope equipped with a ×40 objective and a 10 × 10 eye piece incorporating a graticule. The apoptotic index was expressed as a percentage calculated from the number of cells that stained brown and displayed apoptotic bodies of 3000 tumor cells per section counted under high power, excluding any areas of necrosis.

RESULTS

***In Vitro* Growth Inhibition and Prelamin A Levels Induced by R115777.** R115777 inhibited growth in MCF-7 cells *in vitro* at submicromolar concentrations (Fig. 1*A*). The IC_{50} for growth inhibition was 0.31 ± 0.25 μM. This was in the mid-range of sensitivity for a panel of human tumor cell lines, *e.g.*, compared with IC_{50}s of 1.2 ± 0.1 μM in HT29 colon carcinoma and 0.02 ± 0.011 μM in A2780 ovarian carcinoma cells. Compared with typical dose-response curves obtained previously for cytotoxic drugs in these cell lines, *e.g.*, with cisplatin (26), the dose-response curve for R115777 was relatively flat with 25–75% inhibition occurring across almost 3 logs of concentration (10 nM to 5 μM). There was also some evidence to suggest two phases of growth inhibition: one phase at concentrations up to 100 nM, which led to ~50% growth inhibition, and a second phase at concentrations of ~5 μM, which inhibited growth up to 90%.

We next determined the dose-response relationship in MCF-7 cells for the appearance of prelamin A. Exposure for 24 h to R115777 at concentrations of 0.2–10 μM resulted in the accumulation of prelamin A at concentrations ≥1 μM (Fig. 1*B*). The kinetics of this increase was studied by exposing cells to 2 μM (*i.e.*, approximately six times the IC_{50}) and determining prelamin A levels from 1 to 72 h after the start of continuous exposure to compound. Results showed a peak in levels at 2 h, which was maintained throughout the time course up to 72 h (Fig. 1*C*).

***In Vivo* Antitumor Effects and Pharmacodynamic Markers of Response.** We studied the *in vivo* antitumor activity of R115777 in mice bearing s.c. MCF-7 breast cancer xenografts, using twice daily oral dosing for 10 consecutive days (Fig. 2). Dosing did not commence until tumors had reached diameters of ~7 mm. Results showed that each dose of R115777 (25, 50, and 100 mg/kg) induced some slowing in the rate of growth of the tumors relative to controls treated with drug vehicle alone. In contrast to our previously performed xenograft experiments with cytotoxic drugs such as cisplatin (see *e.g.*, Ref. 27), there was no evidence of tumor shrinkage to less than the starting volume with any dose of R115777. Instead there was a cytostatic effect with relatively little tumor growth over the 10 days during which the drug was administered, followed by recovery toward the control growth rate after dosing was completed. There was some evidence of a dose response in that the lowest level of activity was observed at the 25 mg/kg dose (% T/C at day 21 = 63%; growth delay, 3.6 days) with greater activity observed at the 50 mg/kg dose (% T/C at day 21 = 38%; growth delay, 9.6 days). However, there was no additional gain in inhibition with the highest dose, 100 mg/kg (% T/C at day 21 = 43%).

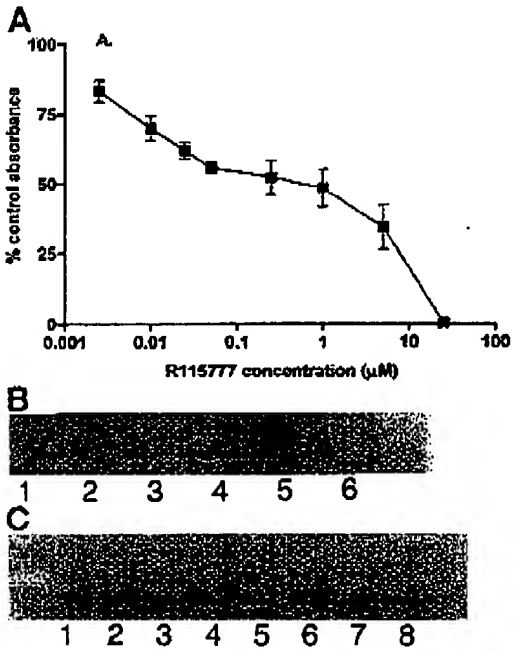

Fig. 1 *In vitro* effects of R115777 against MCF-7 human breast cancer cells. *A*, growth inhibition after 4 days of exposure and assessment of cell number by the SRB assay. Values are mean ± SD (*bars*); $n = 3$. *B*, induction of prelamin A as determined by Western blotting of MCF-7 cells exposed to different concentrations of R115777 for 24 h. *Lane 1*, 0.2 μM; *Lane 2*, 0.4 μM; *Lane 3*, 1.0 μM; *Lane 4*, 2.0 μM; *Lane 5*, 4.0 μM; *Lane 6*, 10 μM. *C*, induction kinetics for prelamin A following exposure of MCF-7 cells to 2 μM R115777. *Lane 1*, 1 h; *Lane 2*, 2 h; *Lane 3*, 4 h; *Lane 4*, 8 h; *Lane 5*, 16 h; *Lane 6*, 24 h; *Lane 7*, 48 h; *Lane 8*, 72 h.

Having observed an increase in prelamin A levels *in vitro* in MCF-7 cells exposed to R115777, we also investigated prelamin A levels 24 h following the final dose (day 11) in tumors excised from mice. Data showed a clear dose-dependent increase in levels of prelamin A in treated tumors (Fig. 3). There was strong accumulation in all five tumors removed from mice treated with the highest dose (100 mg/kg), induction in four of five of tumors exposed to 50 mg/kg, and low-level induction in

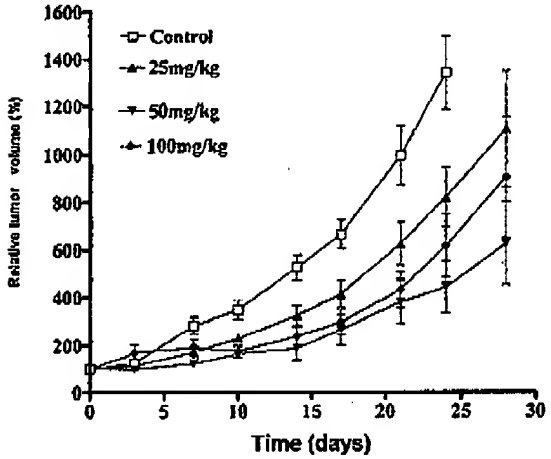

Fig. 2 Tumor growth curves for MCF-7 cells treated with either drug vehicle (*Control*; □) or with varying doses of R115777 administered p.o. twice a day for 10 consecutive days (▲, 25 mg/kg; ▼, 50 mg/kg; ♦, 100 mg/kg). Values are mean ± SD (*bars*); $n = 6$.

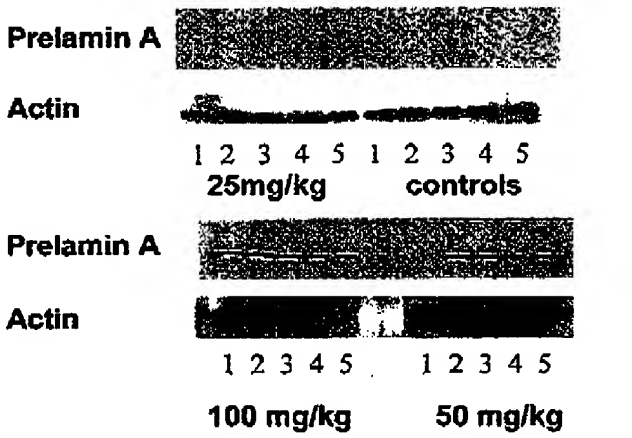

Fig. 3 Increases in prelamin A as determined by Western blotting of MCF-7 xenografts taken at day 11, one day after the final dose of either vehicle (*controls*) or R115777 administered p.o. twice daily for 10 consecutive days at dose of 25, 50, and 100 mg/kg. *Lanes 1–5* for each group refer to individual tumors/animals. Levels of *actin* are shown as loading controls.

two of five tumors exposed to the lowest dose (25 mg/kg). However, there was no prelamin A detectable in any of the five tumors removed from animals dosed with vehicle alone. In contrast, efforts to detect the inhibition of farnesylation of other proteins, such as lamin B, HSP40, or N-Ras, did not produce differences between tumors removed from treated (25, 50, and 100 mg/kg) *versus* control animals (data not shown).

In addition, we investigated markers of tumor proliferation (Ki-67 staining) and apoptosis (TUNEL staining and induction of p21), using control and MCF-7 xenografts treated for 10 days with 100 mg/kg R115777 (Fig. 4, *A–C*). The results showed that R115777 induced a significant 1.5-fold reduction in proliferation (percentage of Ki-67-positive cells; $P = 0.003$). There was also a concomitant increase in apoptosis (percentage of TUNEL-positive cells); this increase was statistically significant compared with controls ($P = 0.007$). Levels of the cyclin-dependent kinase inhibitor p21/CIP1/WAF1 also increased (percentage of positive cells) significantly ($P = 0.024$). In a dose-related pharmacodynamic marker experiment investigating apoptosis at all three R115777 doses, apoptosis increased significantly at the 50 and 100 mg/kg levels ($P = 0.027$ and 0.035, respectively) but did not attain statistical significance at the lower (25 mg/kg) dose (Fig. 4*D*).

We also determined, in a pilot study, whether any changes in prelamin A expression were detectable in the PBLs of four patients with breast cancer from our Phase II clinical trial (23). Samples were collected before the start of treatment and after patients received 300 mg of oral R115777 twice daily every day for 4 weeks. In two nonresponding patients, there was no detectable prelamin A in either pre- or posttreatment PBLs. In another nonresponding patient (Fig. 5, *Patient 2*), there were also no changes in prelamin A levels. By contrast, compared with a blood sample collected prior to the start of treatment, there was clear increase in prelamin A in the 4-week PBL sample of a patient (Fig. 5, *Patient 1*) who showed an objective response to R115777.

DISCUSSION

R115777 represents one of the first-in-class inhibitors of FPT to enter the clinic (6). In a Phase I clinical trial using oral dosing twice daily for 5 consecutive days every 2 weeks, dose-limiting toxicities of neuropathy (one patient) and fatigue were observed. Although initially developed as Ras inhibitors, it is now apparent that the *in vitro* and *in vivo* antitumor effects of drugs such as R115777 may be attributable to effects on a variety of proteins that require post-translational modification by prenylation. Our studies were performed in association with an ongoing Phase II clinical trial in patients with breast cancer (23). The aims were to determine the level of *in vitro* and *in vivo* antitumor activity of R115777 in the MCF-7 estrogen receptor-positive, wild-type Ras, wild-type p53 model of breast cancer and to identify possible pharmacodynamic markers of response mediated via inhibition of protein prenylation. Our Phase II breast cancer trial involved oral dosing with 300 mg of R115777 twice daily in a total of 41 patients; objective responses (tumor shrinkage of at least 50%) were seen in 4 patients (10%) with an additional 6 patients (15%) having stabilization of disease for at least 6 months (range, 6–12+ months; Ref. 23).

Our results reveal that submicromolar concentrations of R115777 (>50 nM) induce significant growth inhibition of MCF-7 cells *in vitro* when added to cells continuously for 4 days, as assessed by cell numbers in the SRB assay. The dose-response curve for growth inhibition is relatively flat and possibly biphasic compared with those that we obtained using MCF-7 cells and the SRB assay with cytotoxic drugs (*e.g.*, cisplatin).[3] The IC_{50} of 310 nM for R115777 is somewhat higher than values reported recently for a panel of ~30 cell lines,

[3] Unpublished data.

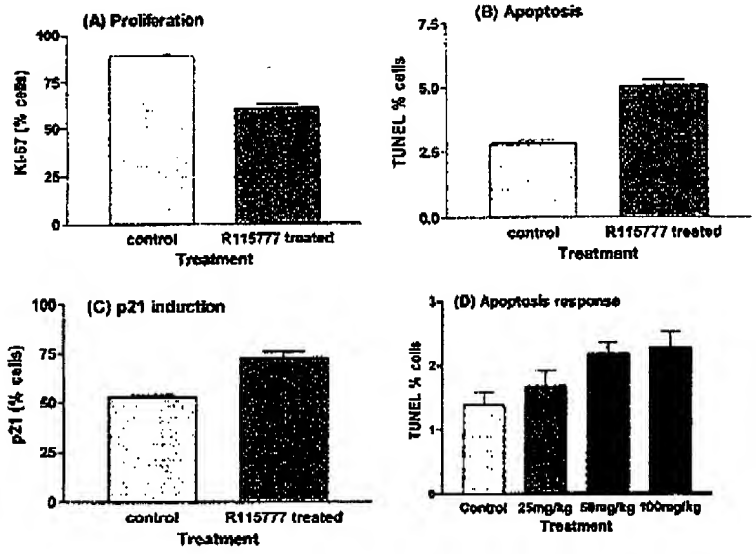

Fig. 4 Immunohistochemical analyses of proliferation (based on Ki-67 staining; *A*), of apoptosis (based on TUNEL staining; *B*), and of p21 induction (*C*) in MCF-7 xenografts removed from vehicle-treated (*control*) or R115777-treated (100 mg/kg administered p.o. twice daily for 10 consecutive days) animals. *D*, apoptosis as determined by TUNEL staining in either MCF-7 xenografts removed from vehicle-treated (*Control*) or R115777-treated (25, 50, or 100 mg/kg administered p.o. twice daily for 10 consecutive days) animals. Values are mean ± SD (*bars*); $n = 5$ tumors.

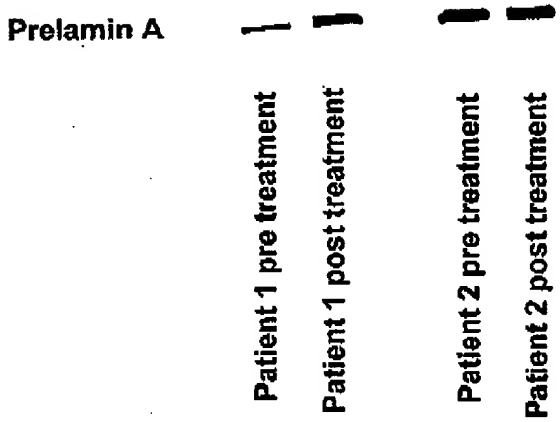

Fig. 5 Increases in prelamin A in patient PBL samples before and after treatment with R115777 (samples collected after patients had received 300 mg twice daily every day for 4 weeks) in *Patient 1* (who exhibited a partial response) and *Patient 2* (who did not respond). In two patients (nonresponders), there was no detectable prelamin A in either sample.

where IC_{50}s of 1–50 nM were obtained for a number of lines (14). However, that study used a different cell proliferation assay and a 4–7 day assay end point. It should also be noted that our *in vitro* MCF-7 studies did not use any added estrogen but relied on provision from FCS. Pharmacokinetic studies performed as part of the oral-dosing Phase I trial showed that peak plasma R115777 concentrations of ~2 μg/ml occurred 0.5–4 h post administration of 325 mg of drug (6). Similar levels were obtained after the first dose on day 1 and after the last dose on day 6.

At concentrations >1 μM (after 24 h of drug exposure), there was evidence of inhibition of prenylation, detected as the appearance of unprocessed prelamin A by an antibody that recognizes the COOH-terminal domain. This precursor of the intranuclear intermediate filament protein lamin A has been shown to require prenylation for processing. The processing involves removal of a 13-amino acid peptide from the COOH terminus of prelamin A and is dependent on farnesylation (25). Moreover, prelamin A has recently been shown to increase in cancer cells (including MCF-7) exposed *in vitro* to FTIs, including R115777 (24). In MCF-7 cells, the appearance of prelamin A occurred at concentrations similar to those required for growth inhibition of the MCF-7 cell line in continuous exposure experiments. Also of note in relation to monitoring inhibition of protein prenylation *in vivo* is that our *in vitro* data obtained from MCF-7 cells exposed to 2 μM R115777 indicate that prelamin A appeared within 2 h and remained detectable for up to 72 h, the longest time point studied.

Notably, R115777 induced a significant antitumor effect against MCF-7 tumors grown s.c. in immune-suppressed nude mice. Three significant points may be derived from this experiment. (*a*) Antitumor activity was obtained with an established s.c. (advanced stage) model of breast cancer, tumors being an average of ~7 mm in diameter at the onset of treatment. In previously reported experiments where R115777 induced an antitumor effect in s.c. human tumor xenografts (CAPAN-2 pancreatic, LoVo colon, and C32 melanoma; Ref. 14), treatment began only 3 days after tumor cell inoculation. (*b*) Compared with previously obtained data in xenograft models using cisplatin (27), the effect of R115777 was mainly to slow the rate of tumor growth relative to controls rather than to induce a cytotoxic reduction in tumor volume. These tumor inhibition data are more consistent with those that we obtained in MCF-7 xenografts with antiendocrine drugs (29, 30). As a consequence of this predominantly cytostatic antitumor effect, a probable clinical scenario is the use of R115777 in combination with cytotoxic chemotherapeutics such as paclitaxel. Of note are

preclinical studies (including in xenografts) in which, when combined, FTIs (including R115777) and paclitaxel produced additive synergistic antitumor effects (32–37). (*c*) In this model, the antitumor effect of R115777 was slightly greater at a dose of 50 *versus* 25 mg/kg but did not improve further at the highest dose, 100 mg/kg. This may be as a result of either a "threshold" effect for the inhibition of prenylation or, because of saturable drug pharmacokinetics, limited amounts of drug reaching the tumor at high doses. This was also matched by a dose-threshold effect on apoptosis. Interestingly, it has been reported that for mice bearing A549 non-small cell lung cancer xenografts treated with FTI-2148, complete inhibition of farnesyl transferase itself is not necessary to induce inhibition of tumor growth (10).

Our pharmacodynamic marker investigations indicate that, of the four proteins studied *in vivo*, the appearance of prelamin A appeared to be most sensitive to the antitumor effects of R115777. Three other proteins known to be prenylated (N-Ras, lamin B, and HSP40; Ref. 10) did not show differences between MCF-7 xenografts removed from R115777-treated and untreated control animals. This may have been because of the relative quality of the antibodies (and because an increase in prelamin A protein, not a decrease in signal, was being detected) or may represent a real biological effect whereby the effects on prelamin A are more pronounced than the effects on the remaining farnesylated proteins. These data provide evidence of inhibition of prenylation *in vivo* that broadly correlated with tumor inhibition and suggest that monitoring the appearance of prelamin A as part of clinical trials with R115777 may provide a sensitive and convenient pharmacodynamic marker of inhibition. Furthermore, our pilot study using four paired samples suggests that it may be feasible to use PBLs as a surrogate tissue; we observed an increase in prelamin A expression in a responding but not a nonresponding patient when we compared posttreatment PBL samples with pretreatment PBL samples. In this context, in a recently reported Phase I trial with the FTI SCH66336 (5), the appearance of prelamin A as an indicator of inhibition of farnesylation was observed in buccal mucosa cells of treated patients.

We have extensive prior experience determining effects on proliferation (using Ki-67) and apoptosis in xenografts, especially MCF-7 treated with antiendocrine drugs (29, 30), and in clinical breast cancer biopsies (38). The results from the TUNEL assay revealed that R115777 induces a significant increase in apoptotic index *in vivo* in s.c. MCF-7 tumors. A similar modest but significant increase in apoptosis has also been reported in mice bearing CAPAN-2 pancreatic xenografts treated with 100 mg/kg oral R115777 (14). A higher level of apoptosis was observed in another tumor (C32 melanoma) as part of the same study. Data suggest that the PI3 kinase/AKT pathway may be a critical target for FTI-induced apoptosis in cell lines (20, 39). These data suggest that the antitumor effects of R115777 observed in MCF-7 xenografts may not be entirely cytostatic in nature, but that some dose-related apoptosis may contribute as well. In addition, a significant decrease in proliferation, evident from staining for Ki-67 and concomitant with an increase in the cell cycle-dependent kinase inhibitor $p21^{CIP1/WAF1}$, was observed. By contrast, no changes in p21 levels were observed in cell lines, including MCF-7, exposed to the FTI SCH66336 (24).

Future studies will seek to identify changes in prelamin A in post- *versus* pretreatment tumor biopsy material collected as part of the breast cancer trial. In addition, gene expression profiling, as we described recently for the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (40), is being used with tumor biopsies from breast cancer patients receiving R115777. This may identify additional sensitive markers of response and shed further light on how R115777 induces antitumor effects (*e.g.*, the possible role of the PI3 kinase/AKT pathway). Furthermore, there is some evidence to suggest that R115777 may also induce antitumor effects *in vivo* by an antiangiogenic mechanism involving endothelial cells, vascular endothelial growth factor, and factor VIII (14).

In summary, R115777 is a potent inhibitor of cell growth in MCF-7 breast cancer cells; it also induced significant cytostatic antitumor effects in a companion s.c. advanced stage breast cancer xenograft model when administered p.o. twice a day for 10 consecutive days. The *in vivo* antitumor effect was associated with a significant dose-related induction of apoptosis. The appearance of unprocessed prelamin A may provide a sensitive pharmacodynamic marker of response for further clinical studies of R115777 in breast and other cancers.

REFERENCES

1. Casey, P. J., Solski, P. A., Der, C. J., and Buss, J. E. p21ras is modified by a farnesyl isoprenoid. Proc. Natl. Acad. Sci. USA, *86:* 8323–8327, 1989.

2. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. All *ras* proteins are polyisoprenylated but only some are palmitoylated. Cell, *57:* 1167–1177, 1989.

3. Kohl, N. E., Mosser, S. D., deSolms, S. J., Giuliani, E. A., Pompliano, D. L., Graham, S. L., Smith, R. L., Scholnick, E. M., Oliff, A., and Gibbs, J. B. Selective inhibition of *ras*-dependent transformation by a farnesyltransferase inhibitor. Science (Wash. DC), *260:* 1934–1942, 1993.

4. Kohl, N. E., Omer, C. A., Conner, M. W., Anthony, N. J., Davide, J. P., deSolms, S. J., Giuliani, E. A., Gomez, R. P., Graham, S. L., Hamilton, K., Handt, L. K., Hartman, G. D., Koblan, K. S., Kral, A. M., Miller, P. J., Mosser, S. D., O'Neill, T. J., Rands, E., Schaber, M. D., Gibbs, J. B., and Oliff, A. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in *ras* transgenic mice. Nat. Med., *1:* 792–797, 1995.

5. Adjei, A. A., Erlichman, C., Davis, J. N., Cutler, D. L., Sloan, J. A., Marks, R. S., Hanson, L. J., Svingen, P. A., Atherton, P., Bishop, W. R., Kirschmeier, P., and Kaufmann, S. H. A Phase I trial of the farnesyl transferase inhibitor SCH66336: evidence for biological and clinical activity. Cancer Res., *60:* 1871–1877, 2000.

6. Zujewski, J., Horak, I. D., Bol, C. J., Woestenborghs, R., Bowden, C., End, D. W., Piotrovsky, V. K., Belly, C. R. T., Todd, A., Kopp, W. C., Kohler, D. R., Chow, C., Noone, M., Hakim, F. T., Larkin, G., Gress, R. E., Nussenblatt, R. B., Kremer, A. B., and Cowan, K. H. Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. J. Clin. Oncol., *18:* 927–941, 2000.

7. Schellens, J. H., de Klerk, G., Swart, M., Palmer, P. A., Bol, C. J., van't Veer, L. J., Tan, S., de Gast, G. C., Beijnen, J. H., and ten Bokkel Huinink, W. W. Phase I and pharmacologic study with the novel farnesyltransferase inhibitor (FTI) R115777. Proc. Am. Soc. Clin. Oncol., *19:* 184, 2000.

8. Eskens, F. A. L. M., Stoter, G., and Verweij, J. Farnesyl transferase inhibitors: current developments and future perspectives. Cancer Treat. Rev., *26:* 319–332, 2000.

9. Johnston, S. R. D. Farnesyl transferase inhibitors: a novel targeted therapy for cancer. Lancet Oncol., *2:* 18–26, 2001.

10. Sebti, S. M., and Hamilton, A. D. Farnesyltransferase and geranylgeranyltransferase I inhibitors in cancer therapy: important mechanistic and bench to bedside issues. Exp. Opin. Investig. Drugs, *9:* 2767–2782, 2000.

11. End, D. W., Skrzat, S., Devine, A., Angibaud, P., Venet, M., Sanz, G., and Bowden, C. R115777, a novel imidazole farnesyl protein transferase inhibitor (FTI): biochemical and cellular effects in H-ras and K-ras dominant system. Proc. Am. Assoc. Cancer Res., *39:* 270, 1998.

12. Skrzat, S., Angibaud, P., Venet, M., Sanz, G., Bowden, C., and End, D. W. R115777, a novel imidazole farnesyl protein transferase inhibitor (FTI) with potent oral antitumor activity. Proc. Am. Assoc. Cancer Res., *39:* 317, 1998.

13. Smets, G., Xhonneux, B., Cornelissen, F., End, D. W., Bowden, C., and Wouters, W. R115777, a selective farnesyl protein transferase inhibitor (FTI), induces anti-angiogenic, apoptotic and anti-proliferative activity in CAPAN-2 and in LoVo tumor xenografts. Proc. Am. Assoc. Cancer Res., *39:* 318, 1998.

14. End, D. W., Smets, G., Todd, A., Applegate, T. L., Fuery, C. J., Angibaud, P., Venet, M., Sanz, G., Poignet, H., Skrzat, S., Devine, A., Wouters, W., and Bowden, C. Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 *in vivo* and *in vitro*. Cancer Res., *61:* 131–137, 2001.

15. Clark, G. J., and Der, C. J. Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res. Treat., *35:* 133–144, 1995.

16. Cox, A. D., and Der, C. J. Farnesyltransferase inhibitors and cancer treatment: targeting simply ras? Biochem. Biophys. Acta, *1333:* F51–F71, 1997.

17. Lebowitz, P. F., Casey, P. J., Prendergast, G. C., and Thissen, J. A. Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J. Biol. Chem., *272:* 15591–15594, 1997.

18. Kodaki, T., Woscholski, R., Hallberg, B., Rodriguez-Viciana, P., Downward, J., and Parker, P. J. The activation of phosphatidylinositol 3-kinase by ras. Curr. Biol., *4:* 798–806, 1994.

19. Yan, J., Roy, S., Apolloni, A., Lane, A., and Hancock, J. F. Ras isoforms vary in their ability to activate raf-1 and phosphoinositide 3-kinase. J. Biol. Chem., *273:* 24052–24056, 1998.

20. Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S. V., Hamilton, A. D., Sebti, S. M., and Cheng, J. Q. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. Mol. Cell. Biol., *20:* 139–148, 2000.

21. Rowell, C. A., Kowalczyk, J. J., Lewis, M. D., and Garcia, A. M. Direct demonstration of geranylgeranylation and farnesylation of ki-ras *in vivo*. J. Biol. Chem., *272:* 14093–14097, 1997.

22. Whyte, D. B., Kirschmeier, P., Hockenberry, T. N., Nunez-Oliva, I., James, L., Catino, J. J., Bishop, W. R., and Pai, J-K. K- and N-ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J. Biol. Chem., *272:* 14459–14464, 1997.

23. Johnston, S. R. D., Hickish, T., Ellis, P. A., Houston, S., Howes, A., Dowsett, M., and Palmer, P. Clinical activity with the farnesyl transferase inhibitor R115777 in patients with advanced breast cancer-relationship with tumour phenotype. Breast Cancer Res. Treat., *64:* 32, 2000.

24. Adjei, A. A., Davis, J. N., Erlichman, C., Svingen, P. A., and Kaufmann, S. H. Comparison of potential markers of farnesyltransferase inhibition. Clin. Cancer Res., *6:* 2318–2325, 2000.

25. Beck, L. A., Hosick, T. J., and Sinensky, M. Isoprenylation is required for the processing of the lamin a precursor. J. Cell Biol., *110:* 1489–1499, 1990.

26. Holford, J., Sharp, S. Y., Murrer, B. A., Abrams, M., and Kelland, L. R. *In vitro* circumvention of cisplatin-resistance by the novel sterically hindered platinum complex AMD473. Br. J. Cancer, *77:* 366–373, 1998.

27. Kelland, L. R., Jones, M., Abel, G., Valenti, M., Gwynne, J. J., and Harrap, K. R. Human ovarian carcinoma cell lines and companion xenografts: a disease oriented approach to new platinum anticancer drug development. Cancer Chemother. Pharmacol., *30:* 43–50, 1992.

28. Sharp, S. Y., Smith, V., Hobbs, S., and Kelland, L. R. Lack of a role for MRP1 in platinum drug resistance in human ovarian cancer cell lines. Br. J. Cancer, *78:* 175–180, 1998.

29. Detre, S., Salter, J., Barnes, D. M., Riddler, S., Hills, M., Johnston, S. R. D., Gillett, C., A'Hern, R., and Dowsett, M. Time-related effects of estrogen withdrawal on proliferation- and cell death-related events in MCF-7 xenografts. Int. J. Cancer, *81:* 309–313, 1999.

30. Johnston, S. R. D., Boeddinghaus, I. M., Riddler, S., Haynes, B. P., Hardcastle, I. R., Rowlands, M., Grimshaw, R., Jarman, M., and Dowsett, M. Idoxifene antagonizes estradiol-dependent MCF-7 breast cancer xenograft growth through sustained induction of apoptosis. Cancer Res., *59:* 3646–3651, 1999.

31. Mainwaring, P. N., Ellis, P. A., Detre, S., Smith, I. E., and Dowsett, M. Comparison of *in situ* methods to assess DNA cleavage in apoptotic cells in patients with breast cancer. J. Clin. Pathol., *51:* 34–37, 1998.

32. Shi, B., Gurnani, M., Yaremko, B., Lee, S., Chen, J., Lipari, P., Ferrari, E., Malkowski, M., Liu, M., Gerald Haijan, G., and Nielsen, L. L. Enhanced efficacy of the farnesyl protein transferase inhibitor SCH66336 in combination with paclitaxel. Proc. Am. Assoc. Cancer Res., *40:* 524, 1999.

33. Skrzat, S. G., Bowden, C. R., and End, D. W. Interaction of the farnesyl protein transferase inhibitor (FTI) R115777 with cytotoxic chemotherapeutics *in vitro* and *in vivo*. Proc. Am. Assoc. Cancer Res., *40:* 523, 1999.

34. Sun, J., Blaskovich, M. A., Knowles, D., Qian, Y., Ohkanda, J., Bailey, R. D., Hamilton, A. D., and Sebti, S. M. Antitumor efficacy of a novel class of non-thiol-containing peptidomimetic inhibitors of farnesyltransferase and geranylgeranyltransferase I: combination therapy with the cytotoxic agents cisplatin, Taxol and gemcitabine. Cancer Res., *59:* 4919–4926, 1999.

35. Verschraegen, C. F., Hu, W., Freedman, R. S., Kudelka, A. P., Kavanagh, J. J., and Yeung, S-C. J. Growth inhibitory effect of FTI alone and in combination with quercetin on ovarian cancer cell lines. Proc. Am. Assoc. Cancer Res., *42:* 487, 2001.

36. Kim, E. S., Glisson, B. S., Meyers, M. L., Herbst, R. S., Shin, D. M., Statkevich, P., Bangert, S., Hong, W. K., and Khuri, F. R. A Phase I/II study of the farnesyl transferase inhibitor (FTI) SCH66336 with paclitaxel in patients with solid tumors. Proc. Am. Assoc. Cancer Res., *42:* 488, 2001.

37. Patnaik, A., Izbicka, E., Eckhardt, S. G., Davidson, K., Goetz, A., McCreery, H., Tolcher, A., Mori, M., Terada, K., Bol, K., Ryback, M., Thibault, A., Richards, H., Gentner, L., and Rowinsky, E. Inhibition of HDJ2 protein farnesylation in peripheral blood mononuclear cells as a pharmacodynamic endpoint in a Phase I study of R115777 and gemcitabine. Proc. Am. Assoc. Cancer Res., *42:* 488, 2001.

38. Chang, J., Ormerod, M., Powles, T. J., Allred, D. C., Ashley, S. E., and Dowsett, M. Apoptosis and proliferation as predictors of chemotherapy response in patients with breast carcinoma. Cancer (Phila.), *89:* 2145–2152, 2000.

39. Du, W., Liu, A., and Prendergast, G. C. Activation of the PI3'K-AKT pathway masks the proapoptotic effects of Farnesyltransferase inhibitors. Cancer Res., *59:* 4208–4212, 1999.

40. Clarke, P. A., Hostein, I., Banerji, U., Di Stefano, F., Maloney, A., Walton, M., Judson, I., and Workman, P. Gene expression profiling of human colon cancer cells following inhibition of signal transduction by 17-allylamino-17-demethoxygeldanamycin, an inhibitor of the hsp90 molecular chaperone. Oncogene, *19:* 4125–4133, 2000.

Phase I and Pharmacokinetic Study of Farnesyl Protein Transferase Inhibitor R115777 in Advanced Cancer

By J. Zujewski, I.D. Horak, C.J. Bol, R. Woestenborghs, C. Bowden, D.W. End, V.K. Piotrovsky, J. Chiao, R.T. Bell, A. Todd, W.C. Kopp, D.R. Kohler, C. Chow, M. Noone, F.T. Hakim, G. Larkin, R.E. Gress, R.B. Nussenblatt, A.B. Kremer, and K.H. Cowan

Purpose: To determine the maximum-tolerated dose, toxicities, and pharmacokinetic profile of the farnesyl protein transferase inhibitor R115777 when administered orally bid for 5 days every 2 weeks.

Patients and Methods: Twenty-seven patients with a median age of 58 years received 85 cycles of R115777 using an intrapatient and interpatient dose escalation schema. Drug was administered orally at escalating doses as a solution (25 to 850 mg bid) or as pellet capsules (500 to 1300 mg bid). Pharmacokinetics were assessed after the first dose and the last dose administered during cycle 1.

Results: Dose-limiting toxicity of grade 3 neuropathy was observed in one patient and grade 2 fatigue (decrease in two performance status levels) was seen in four of six patients treated with 1,300 mg bid. The most frequent clinical grade 2 or 3 adverse events in any cycle included nausea, vomiting, headache, fatigue, anemia, and hypotension. Myelosuppression was mild and infrequent. Peak plasma concentrations of R115777 were achieved within 0.5 to 4 hours after oral drug administration. The elimination of R115777 from plasma was biphasic, with sequential half-lives of about 5 hours and 16 hours. There was little drug accumulation after bid dosing, and steady-state concentrations were achieved within 2 to 3 days. The pharmacokinetics were dose proportional in the 25 to 325 mg/dose range for the oral solution. Urinary excretion of unchanged R115777 was less than 0.1% of the oral dose. One patient with metastatic colon cancer treated at the 500-mg bid dose had a 46% decrease in carcinoembryonic antigen levels, improvement in cough, and radiographically stable disease for 5 months.

Conclusion: R115777 is bioavailable after oral administration and has an acceptable toxicity profile. Based upon pharmacokinetic data, the recommended dose for phase II trials is 500 mg orally bid (total daily dose, 1,000 mg) for 5 consecutive days followed by 9 days of rest. Studies of continuous dosing and studies of R115777 in combination with chemotherapy are ongoing.

J Clin Oncol 18:927-941.

THERAPIES DIRECTED against specific molecular targets offer the promise of increased antitumor efficacy with decreased toxicity. The *ras* proto-oncogene encodes a 21-kd guanosine triphosphate–binding protein Ras, which is a critical component in cellular signal transduction associated with cell proliferation, differentiation, and other pleiotropic responses.[1] Activating, oncogenic, point mutations in codons 12, 13, and 61 of the *ras* gene have been observed in approximately 30% of adult human solid tumors, including pancreas, lung, colon, bladder, and other tumors.[1-11] The wild-type Ras protein may also contribute to the growth of tumors that are driven by the aberrant activation of growth factor receptors and other tyrosine-specific protein kinases.[12-16]

To function in signal transduction and malignant transformation, Ras must localize to the plasma membrane.[17-20] Lacking membrane-binding domains, newly synthesized Ras requires sequential posttranslational enzymatic processing before membrane attachment. The initial and rate-limiting step involves the covalent attachment of a 15-carbon farnesyl moiety via a thioether bond to a single cysteine positioned exactly four amino acids from the carboxyl terminus.[21] This reaction is catalyzed by the enzyme farnesyl protein transferase. The C-terminal recognition sequence has become known as a CAAX motif to indicate the requirement for a cysteine followed by two neutral amino acids (A) with a C-terminal serine or methionine for recognition by farnesyl protein transferase. Farnesylation is followed by cleavage of the three terminal amino acids by a CAAX protease.[22] The resulting C-terminal farnesylcysteine moiety is further carboxy-O-methylated to create the proper hydrophobicity or molecular recognition features to allow plasma membrane localization

From the Medicine Branch, Division of Clinical Sciences, National Cancer Institute; Clinical Center, National Institutes of Health; and National Eye Institute, Bethesda; SAIC-Frederick, Frederick, MD; Janssen Research Institute, Titusville, NJ; Janssen Research Foundation, Beerse, Belgium; Ortho-Clinical Diagnostics, Rochester, NY; and Johnson and Johnson Research, Sydney, Australia.

Submitted March 2, 1999; accepted November 3, 1999.

Supported in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-56000.

The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organization imply endorsement by the United States government.

Address reprint requests to Jo Anne Zujewski, MD, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, 9000 Rockville Pike, Bethesda, MD; email zujewski@nih.gov.

0732-183X/00/1804-927

within cells.[22] The delineation and purification of enzymes involved in Ras processing created the opportunity to downregulate *ras* function in tumor cells by preventing proper localization of the protein. The demonstration that activated, oncogenic Ras lacking the c-terminal cysteine lost cell-transforming activity and the description of simple CAAX tetrapeptide inhibitors of the enzyme farnesyl protein transferase focused drug discovery efforts on this posttranslational step.[21,23]

Initial reports on the cellular effects of farnesyl protein transferase inhibitors that were CAAX peptidomimetics suggested that this class of agent selectively reversed the *ras*-transformed phenotype in cell lines bearing *ras* mutations.[24-26] These findings were very promising because polymerase chain reaction (PCR)–based DNA diagnostics were available that would allow the detection of *ras* mutations and possibly the preselection of patients who would be the best candidates for this *ras*-targeted therapy.[27] However, subsequent preclinical studies have shown the pharmacology of farnesyl protein transferase inhibitors to be more complex. First, farnesyl protein transferase inhibitors, including R115777, have shown antiproliferative effects in vitro and antitumor effects in vivo in cell lines with wild-type *ras*.[28-30] The effects of this class of agent are clearly not dependent upon the presence of mutant Ras, although the compounds are highly effective in cell lines transformed by mutant *ras* also.[25,31-34] Also, it was reported that the K-*ras* isoform of Ras had a much higher affinity for farnesyl protein transferase than the H-*ras* or N-*ras* isoform.[35] Inhibitors that were competitive for the Ras substrate-binding site of the enzyme were much less effective in blocking K-*ras* farnesylation in cell-free systems. An additional complicating issue was introduced by the observation that the CVIM CAAX motif of the K-*ras* peptide allowed the molecule to be either farnesylated or geranylgeranylated by geranylgeranyl protein transferase type 1. Geranylgeranyl protein transferase type 1 is quite similar to farnesyl protein transferase but attaches a 20-carbon geranylgeranyl isoprenoid moiety to substrate proteins bearing a CAAX motif with a terminal leucine.[36] In intact cell lines bearing K-*ras* mutations, alternative processing of K-*ras* by the geranylgeranyl protein transferase type 1 pathway was shown to produce resistance to some farnesyl protein transferase inhibitors.[37,38] The results suggested that farnesyl protein transferase inhibitors might be of no practical use in the human tumor setting because K-*ras* mutations account for the vast majority of *ras* mutations in human tumors. However, it has been clearly established that tumors with mutant K-*ras* respond to this class of agent both in vitro and in vivo.[39,40]

Ironically, as the present clinical studies of R115777 and other compounds are being reported, the biochemical basis for the antitumor responses obtained in preclinical models is under intense reevaluation. An emerging hypothesis involving Rho B accounts for some of the discrepancies discussed previously. Like K-*ras*, Rho B can be either farnesylated or geranylgeranylated with isolated enzymes or in intact cells.[41,42] Farnesylated Rho B seems to cooperate in expression of the transformed phenotype downstream of Ras through modulation of cytoskeletal proteins.[43] Expression of constructs of Rho B that can only be geranylgeranylated seems to produce antiproliferative and antitransforming effects that are similar to the effects of farnesyl protein transferase inhibitors.[44] Thus, farnesyl protein transferase inhibitors may produce antitumor effects by altering the balance of farnesylated and geranylgeranylated Rho B in cells.

Although the role of Ras in the antitumor effects of protein farnesyl transferase inhibitors remains ambiguous in preclinical studies, it will be important to assess *ras* gene status in patients entering onto studies of this class of compound. Although the existing data preclude the *ras* gene mutations as an entry criterion for treatment with a farnesyl protein transferase inhibitor, clinical studies may ultimately find a correlation between oncogene status and responses to these compounds. The genetic instability and complexity of human tumor cell lines used for laboratory studies may not be appropriate to the characterization of newer therapies with specific molecular targets.

Regardless of the mechanism, it is clear that this class of compound produces antitumor effects in standard preclinical tumor models, including human tumor xenografts as well as transgenic oncomouse models.[32,44] The crux of modern cancer research is the translation of preclinical antitumor effects into effective clinical therapy. The first step of this translation are the phase I safety and pharmacokinetic evaluations that allow selection of dose and dose schedules for further evaluation. Presented herein are the phase I data for the first farnesyl protein transferase inhibitor to be evaluated in clinical trials, R115777 (Fig 1). R115777 is a substituted quinolone that is a competitive inhibitor of the CAAX peptide-binding site of farnesyl protein transferase.[45] The molecule is an extremely potent inhibitor of farnesylation with isolated enzyme inhibition of lamin B1 (50% inhibitory concentration [IC_{50}], 0.8 nmol/L) and K-*ras* peptide (IC_{50}, 7.9 nmol/L).[40] R115777 is also a potent inhibitor of proliferation of intact cell lines. The IC_{50} required to inhibit H-*ras* transformed fibroblasts is 1.7 nmol/L, and the IC_{50} required to inhibit pancreatic and colon cancer cell lines bearing K-*ras* mutations ranges from 16 to 22 nmol/L.[40] Preclinical studies have demonstrated that R115777 has antitumor effects in murine xenograft models using H-*ras*–transformed fibroblasts[46] and pancreatic and colon cell lines bearing K-*ras* mutations.[30] No

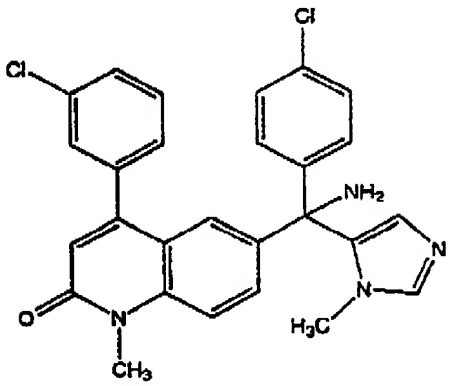

Fig 1. Chemical structure of R115777 or (B)-6-[amino(4-chlorophenyl)(1-methyl-1H-imidazol-5yl)methyl]-4-(3-chlorophenyl)-1-methyl-2(1H)-quinolinone.

gross toxicity to the tumor-bearing host has been observed at effective doses of this compound. After oral administration of R115777 to male Wistar rats and male Beagle dogs, plasma concentrations of R115777 declined with a terminal half-life of less than 1.7 hours and 2.1 hours, respectively. The absolute oral bioavailability was 9% and 66% in the rat and dog, respectively (Janssen Research Foundation, Beerse, Belgium, unpublished observations).

PATIENTS AND METHODS

Patient Eligibility

Patients eligible for this trial had to meet the following criteria: pathologic confirmation of advanced cancer; no available therapy proven to improve survival; last dose of radiation therapy or chemotherapy at least 4 weeks before study entry (6 weeks for nitrosoureas or mitomycin); at least 18 years of age; Zubrod performance status of 0 or 1; adequate hepatic function (normal bilirubin, transaminase levels less than two times the upper limit of normal); normal creatinine levels (0.9 to 1.4 mg/dL for males and 0.7 to 1.3 mg/dL for females); and adequate bone marrow function (absolute neutrophil count $>1,500/\mu L$ and platelet count $>100,000/\mu L$).

Pregnant patients and lactating mothers were ineligible, as were patients with the following characteristics: extensive prior radiation therapy (> 25% of bone marrow reserve); previous bone marrow transplantation or high-dose chemotherapy with bone marrow or stem-cell rescue; untreated CNS metastases; concurrent radiation therapy, chemotherapy, hormonal therapy, or immunotherapy; coexisting medical or psychiatric conditions that were likely to interfere with study procedures; or known allergy to imidazole drugs. All patients were required to provide written informed consent according to National Cancer Institute institutional review board guidelines.

Patient Evaluations

Patient evaluations included the following: complete history and physical examination; complete blood count with leukocyte differential; serum sodium, potassium, chloride, CO_2, blood urea nitrogen, creatinine, calcium, magnesium, total bilirubin, liver transaminases, alkaline phosphatase, lactate dehydrogenase, prothrombin time, partial thromboplastin time, fibrinogen, cholesterol, and triglyceride analyses; urinalysis; pregnancy test (as appropriate); chest radiograph; computed tomography of the chest, abdomen, or pelvis as appropriate; and radionuclide bone scan as appropriate. On-study evaluations included a complete blood count with differential leukocyte count blood chemistry analyses two to three times weekly and radiographic staging studies every 6 weeks (after three 2-week cycles of therapy). Ophthalmologic evaluations, including best visual acuity, ocular history and examination, visual field screening, D-15 color testing, and contrast sensitivity via Pelli-Robson charts (screening tests for abnormalities in visual function[47]), were performed at baseline and during drug administration. Patients with changes in vision that could not be explained by refraction or changes in the anterior segment were to be evaluated with an electroretinogram. This extensive testing was based upon the involvement of several farnesylated proteins on vision[48] and the development of cataracts in one animal species (rats) used in toxicology studies (Janssen, data on file).

Table 1. Dose Escalation Schema

Formulation	Dose Level	Dose Cycle 1 (mg bid × 10 doses)	Dose Cycle 2 (mg bid × 10 doses)	Dose Cycle 3 (mg bid × 10 doses)
Liquid	1	25	50	75
Liquid	2	50	75	125
Liquid	3	75	125	200
Liquid	4	125	200	325
Liquid	5	200	325	525
Liquid	6	325	525	850
Capsule	7	500	800	1,300
Capsule	8	800	800	1,300
Capsule	9	1,300	1,300	1,300

NOTE. Drug was administered orally bid for 10 doses over 5 days every 14 days. The dose level 1 starting dose was 25 mg orally bid (total daily dose, 50 mg) for 5 days.

Treatment Plan

A modified phase I dose escalation schema was used as shown in Table 1. The starting dose was 50 mg daily, less than 1/10th of the lethal dose in dogs. This conservative starting dose was chosen because of the wide interspecies variability in the bioavailability of R115777 (data on file) and the importance of the *ras* signal transduction pathway for normal cellular function. A conservative schedule of R115777 administration was chosen: bid oral administration for 5 days followed by a minimum 7-day period of rest per treatment cycle. This schedule was used because (1) toxicology data in dogs demonstrated hematologic toxicity within 4 days of initiation of dosing with full recovery in 14 days, (2) farnesyl transferase inhibitors had not been tested in humans and acute effects of drug administration were not known, and (3) preclinical pharmacokinetic modeling predicted a half-life of 8 to 12 hours, which would allow for approximately 3 days of steady-state concentrations.

During cycle 1, in order to accommodate 24-hour pharmacokinetic sampling after first-dose administration, a single dose was administered on the first day, followed by bid administration for the next 4 days (days 2 through 5). The final (10th) dose of the cycle was administered on the sixth day.

The schema permitted intrapatient dose escalation, thereby reducing the number of patients who would be treated at potentially subthera-

peutic doses.[49,50] Intrapatient dose escalation to the next dose level was allowed during the second and subsequent treatment cycles provided that nonhematologic toxicity was less than grade 1 in severity, hematologic toxicity was less than grade 2 in severity in the preceding cycle, and no treatment delays were necessary.

Three patients were enrolled at each dose level and observed for at least 14 days before additional patients were entered at the next dose level. If no dose-limiting toxicity was observed in three of three patients at a single dose level, additional patients were entered at the next higher dose level. If dose-limiting toxicity was observed in one patient, additional patients up to a total of six were entered at the same dose level. If two patients developed dose-limiting toxicity at a single dose level, the maximum-tolerated dose was determined to have been exceeded and accrual ceased at that dose level. The first cycle at a new dose level was considered for dose-limiting toxicity (whether entering trial at that dose level or escalating to that dose level). Subsequently, up to a total of six patients could be entered at one dose level below. The maximum-tolerated dose was defined as the highest dose level at which no more than one of six patients experienced a dose-limiting toxicity that could reasonably be attributed to the study drug.

Toxicity

Toxicities were scored according to National Cancer Institute of Canada Clinical Trial Group expanded toxicity criteria. Nonhematologic dose-limiting toxicity was defined as any grade 3 or greater toxicity observed during the first cycle at any dose level (whether entered at that dose level or escalated to that dose level), with the exception of alopecia, nausea, and vomiting. Hematologic dose-limiting toxicity was defined as the occurrence of an absolute neutrophil count less than 500/μL for greater than 3 days or platelet count less than 20,000/μL on a single occasion observed during the first cycle. A treatment delay of more than 3 weeks secondary to toxicity or failure to recover hematologic counts was also considered dose limiting.

Antitumor Response

Patients were evaluated for antitumor response after three cycles of therapy and every three cycles thereafter in selected patients who exhibited some evidence of clinical benefit. A complete response was defined as total disappearance of all clinical evidence of disease for at least two measurements separated by at least 4 weeks. A partial response was defined as at least a 50% reduction in the size of all measurable tumor areas as measured by the sum of the products of the greatest perpendicular, bidirectional measurements without the appearance of new lesions. These parameters must have been present for at least two measurement periods separated by at least 4 weeks. Progressive disease was defined as an increase of more than 25% in measurable disease or the development of new lesions. Stable disease was defined as a tumor status that failed to qualify for either an objective response or progressive disease.

Drug Administration

R115777 was administered orally as an aqueous, cherry-flavored liquid for dose levels 1 through 6 (25 to 850 mg bid). Drug substance was dissolved in a solvent of purified water, hydrochloric acid, benzoic acid, and cherry flavor. For dose levels 7, 8, and 9, (500 to 1,300 mg bid) a hard, gelatin capsule formulation containing 100 mg of R115777/capsule became available and was used. Drug was supplied by the Janssen Research Foundation. Patients were required to fast 1 hour before and 1 hour after administration of R115777.

Pharmacokinetics

During cycle 1, a single dose was administered orally on the morning of the first day to characterize the pharmacokinetics of R115777 for 24 hours after a single oral dose. In addition, R115777 elimination was evaluated for up to 72 hours after the 10th dose given on day 6. Venous blood samples were drawn at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 14, 16, 24, 48, and 72 hours after R115777 administration. Blood samples were also collected during the first cycle on days 3 and 5 just before drug administration to evaluate whether R115777 concentrations had achieved steady state in the blood. For each sample, 7 mL of heparinized blood was collected and immediately placed on ice. Specimens were centrifuged (5 min, 2,500 $\times$ *g*) as soon as possible to collect the plasma and frozen at -70°C until analyzed. Urine was collected on the last day of drug administration during cycle 1 (day 6) to characterize R115777 urinary excretion. A pre-dose urine sample was collected, and a 20-mL aliquot was retained as a pre-dose sample. Patients' complete urinary output during a 12-hour interval was collected. The urine was mixed, the volume and pH were measured, and the sample was frozen at -20°C until assayed. The plasma and urine samples were alkalinized (0.1 mol/L sodium hydroxide), extracted with heptane-isoamyl alcohol (90:10, v/v), and analyzed using a reverse-phase high-performance liquid chromatography column 10 cm $\times$ 4.6 mm internal diameter) packed with 3-μm-particle–sized C18 BDS-Hypersil (Hypersil, Cheshire, United Kingdom). The mobile phase was 0.01 mol/L ammonium acetate-acetonitrile (52:48) at a flow rate of 0.8 mL/min. The chromatographic peaks of R115777 (retention time approximately 4.3 min) and the internal standard (R121550, retention time approximately 6.3 min) were quantified using ultraviolet detection at 240 nm. The mean overall coefficient of variation, as obtained from independently prepared quality control plasma samples, was 6.7% at 14.9 ng/mL, 7.1% at 124 ng/mL, and 7.1% at 2,064 ng/mL. Urine concentrations of R115777 were determined before and after hydrolysis with beta-glucuronidase (from *Escherichia coli*). The validated quantification limit of R115777 in urine was 1.0 ng/mL before hydrolysis and 20 ng/mL after hydrolysis. The following pharmacokinetic parameters were calculated by standard procedures[51]: maximum plasma concentration (C_{max}), time to maximum plasma concentration (t_{max}), minimum concentration in plasma (C_{min}; trough concentration), area under the plasma concentration versus time curve over a 12-hour dosing interval calculated by trapezoidal summation (AUC_{12h}), elimination half-life ($t_{1/2}$), and percentage of dose excreted in the urine. The accumulation ratio was calculated as the AUC_{12h} ratio of day 6 and day 1. The pharmacokinetics of 27 patients were determined. However, the data of one patient at 1,300 mg were incomplete.

To determine the pharmacokinetic parameters that could predict the occurrence of certain adverse events related to the intake of R115777, the following evaluation was performed. The pharmacokinetic parameters C_{max}, AUC_{12}sd (day 1), and AUC_{12}ss (day 6) were tested as predictors for the occurrence of nausea, vomiting, diarrhea, and fatigue. A logistic regression analysis was performed by fitting a generalized linear model with a binary link to the data. The logistic regression model allows the binary data to be converted into a continuous relationship between measures of drug exposure and the probability of developing a certain adverse event. Adverse events were coded as binary response variables (yes or no) without taking into account the severity. The model was parameterized via (i) the predictor value corresponding to 50% probability of having a certain adverse event (P_{50}) and (ii) the sigmoidicity parameter, which reflects the steepness of the probability versus predictor curve (n). The best estimates of parameters and their SEs were obtained via a bootstrap analysis. The

number of bootstrap replications was 1,000. The S-PLUS package (Probability, Statistics, & Information, Seattle, WA) was used throughout the analysis.

Flow Cytometric Analysis

Lymphocyte populations were analyzed by three-color flow cytometry at five time points in the first three cycles of treatment. Peripheral blood, collected in sodium heparin, was stained at ambient temperature with a panel of antibodies, lysed using Optilyse C lysing solution (Coulter Corporation, Opulocka, FL), washed with Dulbecco's phosphate-buffered saline (BioWhittaker, Walkersville, MD), and analyzed on a Coulter XL flow cytometer (Coulter Corporation, Hialeah, FL). Total leukocyte populations and lymphocyte subpopulations were analyzed using the following antibody combinations: IgG1/IgG2a/CD3, CD45/CD14/CD3, CD4/CD8/CD3, CD20/CD19/CD3, and CD3/CD16$^+$CD56/CD8. Commercial sources for antibodies included Becton Dickinson Immunocytometry Systems (Mountainview, CA), Caltag (Burlingame, CA), Pharmingen (San Francisco, CA), Sigma (St Louis, MO), and Immunotech (Marseille, France). B cells were defined as CD3$^-$CD19$^+$CD20$^+$, natural killer cells as CD3$^-$CD16$^+$/CD56$^+$, CD8 cells as CD3$^+$CD8$^+$, and CD4 cells as CD3$^+$CD4$^+$. Proportions of CD45RA and CD45RO (naive *v* activated/memory cells) within the CD4 population were determined by staining cells with three antibodies, acquiring the lymphocyte population, and gating during analysis on CD4$^+$ cells only. Determination of total cells/μL expressing a particular phenotype was calculated by multiplying the total WBC count, as determined using a Coulter counter, by the frequency of that population in the lymphocyte gate and the fraction of total WBCs included in the lymphocyte gate. Lymphocyte populations were compared at the five time points using the Wilcoxon rank test for nonparametric assessments of paired data (Statview 5.0; SAS Institute Inc, Cary, NC).

Analysis of Ras and Prenyl Protein Processing

Lymphocyte samples containing approximately 1×10^7 cells were obtained before administration of R115777 and at the end of the 5-day treatment period. Samples were also obtained from healthy untreated volunteers to control for cell preparation and storage. Lymphocytes were stored frozen as pellets before prenyl protein processing was analyzed essentially as described.[52] Briefly, lymphocyte pellets were resuspended in 0.5 mL of sonication buffer consisting of 20 mmol/L HEPES, 1 mmol/L EDTA, 1 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 μmol/L pepstatin. The cell pellets were lysed by sonication for 20 seconds. The lysates were centrifuged at 100,000 $\times$ *g* for 60 min. The resulting supernatants were transferred to microfuge tubes, and the pellets were resuspended in 0.5 mL of sonication buffer. Protein determinations were performed on 5-μL portions of supernatants and pellets, and samples were diluted to equal protein concentrations in Laemmli sample buffer. Samples (10 to 15 μL) were separated by electrophoresis on 10% to 20% gradient sodium dodecyl sulfate polyacrylamide minigels and transferred to polyvinylidene fluoride membranes. The membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies from Calbiochem (La Jolla, CA) (lamin B1 and pan-Ras Ab-3) and Santa Cruz Biotechnology (Santa Cruz, CA) (Rho B) were used for the Western blot analysis of these prenylated proteins. The immunostained antigens were visualized using horseradish peroxidase–conjugated secondary antibodies and the Amersham ECL–enhanced chemiluminescent detection system (Amersham, Buckinghamshire, United Kingdom).

ras *Mutation Analysis*

Paraffin sections of primary tumors or metastatic sites were analyzed. The first section (4 μm) from each block was stained with hematoxylin and eosin and examined by a pathologist to confirm the presence of cancer and to determine the extent of tumor on the slide. A series of 10-μm sections were cut for mutation analysis. To minimize possible contamination between samples, the microtome was cleaned to remove excess paraffin and a new blade was used between samples. DNA was extracted from one or more of the remaining sections, and samples were analyzed for the presence of *ras* by a nested PCR protocol, followed by restriction fragment length polymorphism (RFLP) analysis. Restriction enzymes were selected such that wild-type *ras* sequences were cleaved, leaving an intact gel band of the expected size when a mutation was present. Cell-line DNA from both wild-type and, where available, mutant *ras* were used as controls. This nested PCR/RFLP method can detect mutations at H-*ras* intron D and all K-, H-, and N-*ras* mutations at codons 12, 13, and 61 (except for H-*ras* codon 13, which is an extremely rare mutation). Studies with cell-line DNA indicated that this protocol detects one mutant allele in a background of 10 wild-type alleles.

RESULTS

Patient Characteristics

Twenty-seven patients were treated in this phase I study. Patient characteristics are listed in Table 2.

Adverse Events

Table 3 includes all adverse events observed during cycle 1 of therapy considered possibly, probably, or very likely related to R115777. Dose-limiting toxicity was observed at the 1,300-mg dose level in one patient who had a prior history of mild peripheral neuropathy attributed to paclitaxel chemotherapy. During cycle 1, she developed severe burning in her lower extremities, oral cavity, and vaginal area. The pain required opioid analgesics and resolved within 24 hours after withholding of the drug. There were no signs of stomatitis or vaginitis on physical examination. The same patient experienced similar but less severe symptoms during her next treatment cycle at a reduced dose (800 mg bid); however, severe (grade 3) symptoms recurred during her third cycle of therapy at 800 mg bid.

Although not defined as dose-limiting, clinically significant fatigue was observed in patients treated at the higher dose levels (800 mg and 1,300 mg bid). With National Cancer Institute of Canada criteria, grade 2 fatigue (two-level decrease in performance status) was observed in one of three patients who received 1,300 mg bid during the first cycle of therapy and in four of six patients treated at 1,300 mg bid during any cycle (Table 4).

One patient developed a grade 2 increase in his serum creatinine level during his second treatment cycle. The patient's baseline creatinine level was 1.1 mg/dL. He received the first cycle of R115777 at 800 mg bid without a

Table 2. Patient Characteristics (n = 27)

		No. of Patients
Age, years		
Median	58	
Range	27-78	
Sex		
Male		13
Female		14
Diagnosis		
Colorectal cancer		11
Breast cancer		7
Other*		9
Prior therapies		
No prior therapy		1
Prior chemotherapy		
1-2 prior regimens		12
≥3 prior regimens		14
Prior hormone/immune therapy		
1 prior regimen		8
2 or more		3
Prior radiation therapy		17
Zubrod performance status		
0		5
1		22
ras mutation status		
K-*ras* codon 12 (colon)		2
K-*ras* codon 61 (liver)		1
Negative		20
Not available		4

*Other diagnoses were rhabdomyosarcoma, non-Hodgkin's lymphoma, adenocarcinoma of unknown primary, sarcoma (not otherwise specified), esophageal carcinoma, gallbladder carcinoma, hepatoma, melanoma, and non-small-cell lung cancer.

significant change in his serum creatinine level. During cycle 2, in which R115777 was administered at 1,300 mg bid, his creatinine level increased to 3.3 mg/dL on day 6. His creatinine level had normalized by day 30. He received cycle 3 at the 800-mg bid dose without event. Evaluation of urine sediment during cycle 2 was remarkable for renal epithelial cells consistent with an acute tubular injury. Proteinuria was not significant. Other causes of renal dysfunction (eg, contrast dye administration, nonsteroidal analgesics, hypotension) and predisposing factors for renal dysfunction were excluded. Eight additional patients were noted to have increased creatinine levels in this study. In five of these eight patients, grade 1 creatinine elevation was noted and considered at least possibly related to R115777 (three patients at the 1,300-mg bid dose level, one patient at the 800-mg bid dose level, and one patient at the 200-mg bid dose level). In one of these five patients, examination of the urinary sediment was also consistent with acute tubular injury during the first cycle at 1,300 mg bid and during a subsequent cycle at 800 mg bid. In three patients, other causes were thought more likely to account for the creatinine elevation (obstruction due to malignant disease in two patients and an inferior vena cava thrombosis in one patient).

Another prominent adverse event was nausea and vomiting. At dose levels 1 through 6, an oral liquid formulation was used. This liquid had an unpleasant taste, and nausea and vomiting were frequently reported. Although the capsule formulation was tolerated better than the liquid formulation, at the highest dose levels, the capsule formulation was also associated with grade 1 and 2 nausea and vomiting. Twenty of 27 patients required antiemetic therapy. The choice of antiemetic was made at the discretion of the prescribing physician. Drugs used included ondansetron, granisitron, prochlorperazine, metoclopromide, lorezepam, and promethazine.

One patient with a baseline history of migraines treated with 125 mg bid experienced a grade 3 headache during her first cycle of therapy. This headache was similar in character but more severe than her prestudy headaches. She was able to continue treatment without subsequent events.

Minimal hematopoietic toxicity was observed in this trial. One patient treated with 50 mg bid experienced grade 3 neutropenia. This patient had multiple prior therapies for breast cancer, including radiation. A review of her complete blood counts obtained before study drug administration demonstrated intermittent grade 3 neutropenia. This patient continued to receive study drug at the same dose level with resolution of her neutropenia.

A second patient with a baseline platelet count of 103,000/μL developed grade 2 thrombocytopenia (72,000/μL) during cycle 1 of R115777 at the 1,300-mg bid dose level. This patient also experienced grade 3 peripheral neuropathy requiring a dose reduction. She was able to continue therapy without delay at the 800-mg bid dose level with resolution of her thrombocytopenia and no subsequent recurrences of thrombocytopenia.

Eight patients required RBC transfusions during this trial. All patients requiring blood transfusions had received prior therapy for their advanced cancer and had multiple blood samples drawn for pharmacokinetic studies and toxicity monitoring.

Several farnesylated proteins are important in maintenance of retinal cytoarchitecture and photoreceptor structure[48]; therefore, all patients were carefully evaluated for ophthalmologic abnormalities. No abnormalities were noted in D-15 color vision and contrast sensitivity testing. Two patients had small unilateral visual field defects while on therapy. In one patient, the visual field defect resolved during continued R115777 therapy. In the second patient, a possible defect in the same area was noted at baseline that became more apparent after initiation of therapy. Both

Table 3. Cycle 1 Toxicities Related to Study Drug by Dose Level

	Dose																											
	25			50			75			125			200			325			500			800			1,300		
	Grade			Grade			Grade			Grade			Grade			Grade			Grade			Grade			Grade		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Nausea	1			2			1			2			1			1			2			2	1		3		
Vomiting				2																		2	1		1		
Fatigue				1						1						1			2			3			2	1	
Lethargy																			1								
Anorexia																			2						1		
Headache				1							1	1				1			1						1		
Hypotension													1												2		
Arthralgia								1								1											
Taste change	2												1														
Heartburn										1			1												2		
Constipation																1											
Hiccoughs										1						1											
Bloating																									2		
Neurocortical													1						1			1			1		
Xerostomia										1																	
Pharyngitis																2											
Chills	1																		1								
Dizziness																			1								
Neuropathy																											1
Creatinine																									2		
Thrombocytopenia																										1	
Neutropenia					1																						
Hemoglobin																	1				1						
Hypomagnesemia																									2		
Hypokalemia																										1	

NOTE. This table includes the number of patients who experienced toxicity, at maximum grade per patient, at each dose level during their first cycle of therapy. Three patients received cycle 1 at each dose level. Toxicities were considered possibly, probably, or very likely related to study drug.

patients were asymptomatic. Retinal examinations were remarkable for the development of abnormalities during drug administration in four patients, including cotton wool spots and small retinal hemorrhages (two patients), small hemorrhage (one patient), and Roth's spots (one patient). These four patients also had a history of diabetes, hypertension, or anemia. All patients were asymptomatic, and the ophthalmologic findings were thought to be consistent with those observable in a chronically ill population. No new or worsening cataracts were noted in this trial.

Several serious adverse events were observed during this trial that were not considered related to the study drug. One patient with history of pulmonary embolism developed an inferior vena cava clot at the site of an inferior vena cava filter. He was taken off study and treated with anticoagulant therapy. One patient with melanoma and a prior history of brain metastasis treated with radiation therapy experienced an unwitnessed seizure. Subsequent magnetic resonance imaging revealed new and enlarged brain metastases. One patient with a history of hypertension experienced an episode of confusion. Imaging studies were consistent with a new small cerebral hemorrhage thought secondary to hypertension. One patient developed a small pericardial effusion and atrial arrythmia thought to be related to progressive malignant disease.

Pharmacokinetics

R115777 was rapidly absorbed, with peak plasma concentrations reached within 0.5 to 3 hours after administration of the oral solution and within 1.5 to 4 hours after administration of the pellet capsules. Pharmacokinetic parameters are shown in Table 5. Representative concentration-time profiles of (mean ± SD) R115777 are shown in Fig 2 for a patient receiving 125 mg bid administered as an oral solution and 500 mg bid administered as a capsule. Within the 25- to 1,300-mg bid dose range, C_{max} values ranged from 93.0 to 3,585 ng/mL on day 1 and from 59.2 to 2,946 ng/mL on day 6, and AUC_{12h} values ranged from 289 to 13,531 ng · h/mL on day 1 and from 315 to 15,724 ng · h/mL on day 6. On day 6, C_{min} values ranged from 6.7 to 363 ng/mL. The elimination of R115777 from plasma was biphasic. The half-life associated with the first elimination

Table 4. Toxicities Related to R115777 During All Cycles by Dose Level (n = 85 cycles)

	Dose															
	50 mg (6 patients, 7 cycles)		75 mg (8 patients, 10 cycles)		125 mg (7 patients, 9 cycles)		200 mg (7 patients, 10 cycles)		325 mg (7 patients, 7 cycles)		500 mg (9 patients, 16 cycles)		800 mg (10 patients, 14 cycles)		1,300 mg (6 patients, 9 cycles)	
	Grade		Grade		Grade		Grade		Grade		Grade		Grade		Grade	
	2	3	2	3	2	3	2	3	2	3	2	3	2	3	2	3
Nausea	1		2					1			2		1	1	1	
Vomiting							2						2	1	1	
Fatigue			2								2		2		4	
Headache					1	1							1			
Hypotension													2		1	
Hypertension								1								
Arthralgia			1													
Myalgia											1					
Edema							1									
Fever							1									
Pericardial			1													
Neuropathy														1		1
Neurocortical								1								
Neutropenia		1														
Hemoglobin	1									1		2	1	1		1
Thrombocytopenia															1	
Thrombocytosis													1		1	
Creatinine															1	
Hypokalemia													2		2	
Hypomagnesmia															1	

NOTE. This table includes the number of patients who experienced toxicities greater than grade 1, at maximum grade per patient, that were considered possibly, probably, or very likely related to study drug, at each dose during all cycles of therapy. The 500-mg level includes toxicities observed in patients receiving the 500-mg capsule formulation or the 525-mg liquid formulation. The 800-mg level includes toxicities observed in patients receiving the receiving 800-mg capsule formulation and the 850-mg liquid formulation of R115777. Patients experiencing toxicities at more than one dose level are reported at all dose levels at which the toxicity was observed. Three patients received three cycles at the 25-mg dose, and there were no grade 3 or 4 adverse events observed.

phase was 5.27 + 3.24 hours (SEM + SD) for the oral solution (n = 17) and 4.34 + 1.4 hours for the pellet capsule (n = 9). The terminal half-life associated with the second phase of elimination varied with the ability to quantify R115777 in plasma. Its median value was about 16 hours. Steady-state conditions were obtained within 2 to 3 days of bid dosing. The accumulation ratio was 1.09 + 0.28 for the oral solution and 0.92 + 0.20 for the capsule and indicates little accumulation of R115777 after a 5-day bid dosing regimen.

Plots of the individual values of C_{max} and AUC_{12h}, evaluated after the first dose on day 1 and the last dose on day 6, versus the administered dose of R115777 (Fig 3) indicate a consistent dose-proportional increase in the 25 to 325-mg dose range for the oral solution. For the capsule, a dose-proportional increase was observed in the 500- to 1,300-mg dose range after the first dose on day 1. However, at day 6, AUC_{12h} and C_{max} seemed to increase less than dose proportional. Since vomiting occurred only during one out of 11 assessments for the pharmacokinetics of the 800- and 1,300-mg doses, it is not likely that the deviation from dose proportionality is the result of drug loss from emesis. The data also suggest that the bioavailability of the capsules is less than that of the oral solution. Furthermore, the data suggest substantial interindividual variability in the oral bioavailability of R115777.

The urinary excretion of unchanged R115777 (n = 15) was negligible, as less than 0.1% of the administered oral dose was excreted in the urine as unchanged drug. In addition, 16.5 ± 12.2% (mean ± SD) of the administered dose was excreted in the urine as the glucuronide conjugate of R115777.

The estimates of the logistic regression model parameters, as listed in Table 6, related frequently observed adverse events with pharmacokinetic parameters. According to the model, fatigue was the only response for which the probability of occurrence could be predicted reliably on the basis of C_{max} and AUCs. The $AUC_{12h}(ss)$ corresponding to a 50% probability to develop fatigue was estimated at 4,210 ± 1,390 ng · h/mL (mean ± SD).

Table 5. Pharmacokinetic Parameters

Parameter	Oral Solution					
	25 mg	50 mg	75 mg	125 mg	200 mg	325 mg
Day 1						
t_{max}, hours	0.8 ± 0.3	1.4 ± 0.6	1.1 ± 0.4	1.5 ± 0.7	1.3 ± 0.3	1.8 ± 1
C_{max}, ng/mL	147 ± 64	266 ± 90	264 ± 140	646 ± 109	712 ± 474	2,045 ± 933
AUC_{12h}, ng · h/mL	470 ± 254	1,005 ± 258	1,021 ± 615	2,475 ± 690	2,616 ± 1,541	8,891 ± 4,045
$t_{1/2}$, hours	5.6 ± 2.0	5.0 ± 1.3	9.1 ± 6.4	4.4 ± 1.9	4.9 ± 1.4	2.7 ± 0.2
Day 6						
t_{max}, hours	1.1 ± 0.4	1.4 ± 0.3	2.2 ± 1.5	1.9 ± 0.3	1.5 ± 0.5	1.8 ± 0.3
C_{min}, ng/mL	10.0 ± 3.0	24.0 ± 15.0	28.0 ± 6.0	84.0 ± 38.0	66.0 ± 46.0	148 ± 89
C_{max}, ng/mL	123 ± 87	285 ± 101	274 ± 51	528 ± 102	778 ± 470	1,549 ± 663
AUC_{12h}, ng · h/mL	506 ± 273	1,108 ± 507	1,207 ± 330	2,537 ± 396	2,940 ± 1,736	7,651 ± 3,070
$t_{1/2terminal}$, hours	12.6 ± 13.8	12.8 ± 8.5	11.5 ± 4.7	20.7 ± 7.5	58.3 ± 48.4	18 ± 7.7
Accum index	1.1 ± 0.2	1.1 ± 0.3	1.3 ± 0.4	1.1 ± 0.3	1.1 ± 0.3	0.9 ± 0.1

Parameter	Capsule		
	500 mg	800 mg	1,300 mg
Day 1			
t_{max}, hours	3.4 ± 0.6	3.0 ± 1.0	1.8 ± 0.3
C_{max}, ng/mL	1,637 ± 996	1,476 ± 569	2,521 ± 1,038
AUC_{12h}, ng · h/mL	9,304 ± 7,669	7,134 ± 2,526	11,970
$t_{1/2}$, hours	3.8 ± 0.5	5.2 ± 1.2	7.1 ± 6.7
Day 6			
t_{max}, hours	3.7 ± 0.6	2.8 ± 1.3	1.6
C_{min}, ng/mL	183 ± 115	187 ± 153	115
C_{max}, ng/mL	1,656 ± 1,124	1,589 ± 969	1,115
AUC_{12h}, ng · h/mL	8,701 ± 6,092	6,951 ± 3,906	5,935
$t_{1/2terminal}$, hours	31.5 ± 17.1	24.6 ± 9.1	13.0
Accum index	1.0 ± 0.2	0.9 ± 0.2	0.6

NOTE. Pharmacokinetic parameters (mean ± SD, n = 3 per dose level or when SD is omitted, n = 2) of R115777 in patients with advanced incurable cancer were evaluated in cycle 1 after the first dose on day 1 and after the last dose on day 6. R115777 was given bid as an oral solution (25 to 325 mg) and as pellet capsules (500 to 1,300 mg).

The model prediction for nausea had a borderline significance. Vomiting and diarrhea could not be related to any of the pharmacokinetic parameters.

Flow Cytometric Analysis

The effect of farnesyltransferase inhibitors on T-, B-, and natural killer–cell populations was assessed by flow cytometry. To assess changes in the threshold for activation of the cells, the expression of early and late activation markers (CD69 and HLA-DR, respectively) and CD45 isoform markers of naive and activated/memory subpopulations was examined in CD4 populations. Finally, atypical CD8 populations expressing CD57 and lacking in CD28 expression expand after chemotherapy or transplantation, in human immunodeficiency virus and in the extreme elderly, in a process that may reflect terminal differentiation of chronically activated cells.[53,54] These CD8 subpopulations were therefore assessed.

Flow cytometric analyses were performed at five time points: pretreatment (cycle 1, day 1), end of the first drug treatment (cycle 1, day 6), end of the first cycle (cycle 1, day 14), end of the second cycle (cycle 2, day 14), and end of the third cycle (cycle 3, day 14). All 27 patients were assessed before the start of therapy, 21 were assessed through the end of two cycles of treatment and recovery, 16 were assessed through three cycles, and two were observed after four and six cycles.

The absolute numbers (cells/μL) of all peripheral-blood lymphocyte populations assessed (CD4, CD8, total CD3, B, and natural killer cells) decreased during the first 5-day treatment period (P[CD4] = .047, P[CD8] = .01, P [natural killer] = .001, P [B] = .36) but recovered to pretreatment levels by the end of cycle 1 or cycle 2. T-cell subsets and B-cell populations at the end of the third cycle (cycle 3, day 14) were reduced 30% to 35% compared with pretreatment levels (P[CD4] = .001, P[CD8] = .001, P[natural killer] = .08, P[B] = .007) but remained within normal adult ranges. The reduction in numbers persisted in the two individuals observed for longer periods. The drop in T and B cells was associated primarily with a decrease in the overall frequency of lymphocytes from an average of 21% ± 2.3% at baseline (cycle 1, day 1) to 16.6% ± 2.3% at cycle 3, day

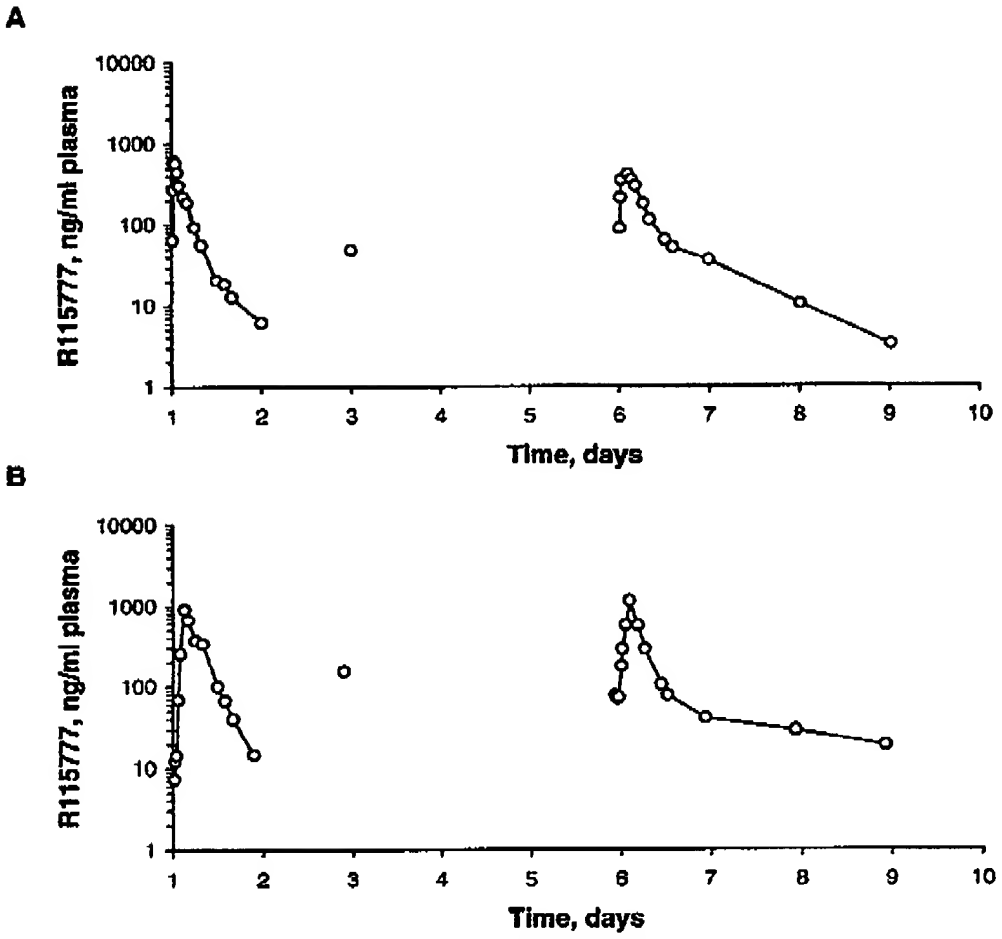

Fig 2. Representative concentration-time profiles of R115777 given bid to patients as an oral solution (A, 125 mg) and as capsules (B, 500 mg).

14; this corresponded to an average decrease in the total number of lymphocytes from 1,454 ± 166 cells/μL to 1,026 ± 114 cells/μL. The total WBC count did not change significantly ($7.3 \pm 0.71 \times 10^3/\mu L$ v $7.1 \pm 0.77 \times 10^3/\mu L$) at these time points. Thus, although a 14-day period was sufficient for recovery of lymphocyte levels after the first two cycles, it was not sufficient for the third.

The percentages of T-cell subsets and B and natural killer cells within the total lymphocyte population remained remarkably constant throughout the study. Furthermore, the frequency of expression of activation markers (HLA-DR), of naive and memory phenotypes in CD4 cells, and of atypical chronically activated CD8 cells remained consistent within each patient. This lack of changes in subpopulations of T cells would be consistent with either altered trafficking of lymphocytes within the peripheral blood or with a nonspecific loss of lymphocyte populations.

Analysis of Ras and Prenyl Protein Processing

The processing of the prenylated protein lamin B1, Ras, and Rho B was studied in lymphocytes using a technique initially developed to study the effects of farnesyl protein transferase inhibitors in tumor cells in tissue culture. The method measures levels of prenylated proteins in particulate membrane fractions and the appearance of unprenylated proteins in the soluble, cytosolic fractions. Strong signals for Rho B and Ras were observed in all particulate membrane fractions. Levels were not decreased by treatment with high doses of R115777 (500, 800, and 1,300 mg).

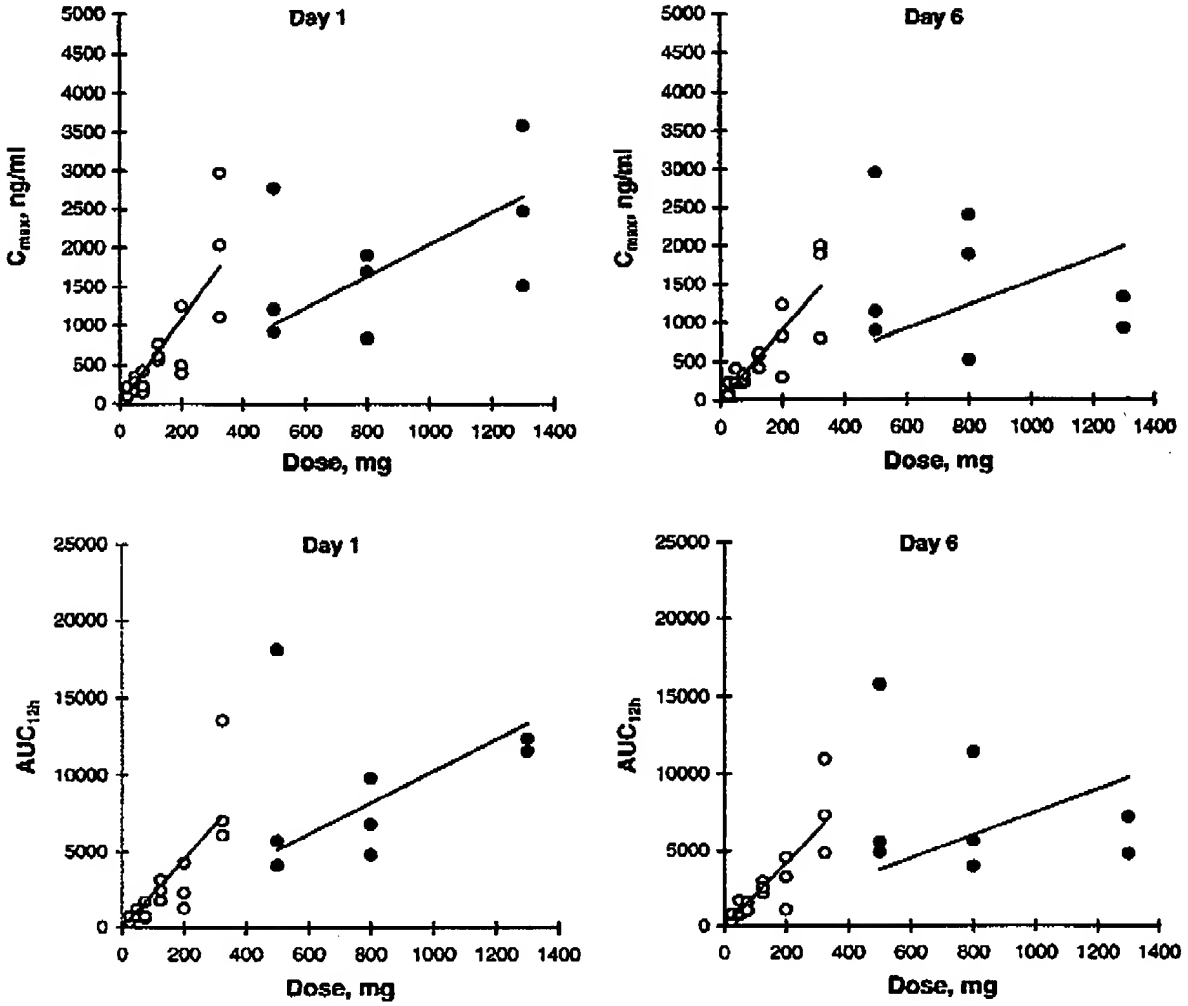

Fig 3. Individual values of C_{max} and AUC_{12h} versus bid dose, evaluated in cycle 1 after the first dose on day 1 and after the last dose on day 6. The oral solution and the oral pellet capsule are represented by open and closed circles, respectively. The trendlines are the regression lines with the intercept set at zero.

There was no appearance of Rho B in soluble fractions after treatment with R115777, an observation consistent with the posttranslational modification of the protein by geranylgeranyltransferase. Evaluation of soluble, unfarnesylated Ras revealed that an antigen appearing randomly in patient and healthy volunteer samples cross-reacted with the pan-Ras antibody. The cross-reactivity seemed to be associated with erythrocyte contamination and hemolysis in samples. This prevented an accurate assessment of soluble unfarnesylated Ras. However, there was no evidence of a treatment-related increase in soluble Ras in the samples. Lamin B1 immunoreactivity was at the limits of detection and could not be reliably measured. It is known that lamin B1 expression is restricted to certain tissues and predominates in proliferating tissues.[55] The low levels of lamin B1 compared with those obtained in cell lines grown in culture may reflect the lack of cell proliferation in the peripheral-blood lymphocyte compartment. Although peripheral-blood lymphocytes may be attractive for monitoring the effects of prenylation inhibitors because of their accessibility, the lack of cell turnover may preclude their utility in biomarker studies.

Table 6. Estimates and SEs of Parameters of the Logistic Model Fitted to All Combinations of Responses and Predictors

Response/Predictor	Units	Parameters	Estimates	SEs	P
Nausea					
C_{max}	ng/mL	P_{50}	384	174	.0371
	-	n	1.21	0.662	.0793
AUC_{12} (sd)	ng · h/mL	P_{50}	1420	676	.0459
	-	n	1.11	0.59	.0723
AUC_{12} (ss)	ng · h/mL	P_{50}	1530	705	.0401
	-	n	1.13	0.667	.104
Vomiting					
C_{max}	ng/mL	P_{50}	1540	2290	.506
	-	n	0.0795	0.427	.854
AUC_{12} (sd)	ng · h/mL	P_{50}	8580	12700	.506
	-	n	0.0835	0.361	.819
AUC_{12} (ss)	ng · h/mL	P_{50}	8650	12800	.505
	-	n	0.223	0.434	.612
Diarrhea					
C_{max}	ng/mL	P_{50}	4370	6480	.506
	-	n	0.343	0.446	.449
AUC_{12} (sd)	ng · h/mL	P_{50}	16800	21600	.443
	-	n	0.433	0.439	.334
AUC_{12} (ss)	ng · h/mL	P_{50}	12400	17200	.478
	-	n	0.466	0.518	.376
Fatigue					
C_{max}	ng/mL	P_{50}	1040	355	.00745
	-	n	1.48	0.611	.0233
AUC_{12} (sd)	ng · h/mL	P_{50}	4210	1390	.00553
	-	n	1.62	0.654	.0205
AUC_{12} (ss)	ng · h/mL	P_{50}	4230	1450	.00717
	-	n	1.54	0.661	.0279

Antitumor Response

Twenty-seven patients were treated on this phase I trial. Two patients did not complete a total of three cycles (6 weeks) of therapy for reasons other than disease progression (one patient for noncompliance and one patient for development of inferior vena cava thrombosis) and were not evaluated for a response. By the end of 6 weeks of therapy, 17 patients had developed progressive disease and therapy was discontinued. Among eight patients who had had stable disease after three cycles of therapy, four continued therapy with R115777 and their disease remained stable for 2 to 5 months. One patient with metastatic colon cancer involving the mediastinum and lung experienced improvement in symptoms (decreased cough) and a carcinoembryonic antigen decrease from 2,991 to 1,626 ng/mL. This patient developed progressive disease after 5 months of R115777 therapy.

DISCUSSION

This phase I trial attempted to determine the maximum-tolerated dose of R115777 when administered orally twice daily for 5 consecutive days followed by 7 to 9 days of rest. The maximum-tolerated dose as defined in the protocol was not reached and dosing was terminated at the highest dose level (1,300 mg bid; total daily dose, 2,600 mg).

Only one dose-limiting toxicity was observed in one of six patients who received R115777 1,300 mg bid. This patient developed grade 3 peripheral neuropathy, described as a painful burning sensation in the extremities, oral cavity, and vaginal area. Although not defined as dose limiting, grade 2 fatigue (decrease in two performance status levels) was observed in four of six patients at the 1,300-mg bid dose level and two of nine patients at the 800-mg bid dose level. Grade 1 to 2 increases in serum creatinine levels and urinary findings consistent with acute tubular injury were noted in two patients treated with the 1,300-mg dose, which suggests that R115777 may be nephrotoxic at high doses. Therefore, we recommend that patients maintain adequate hydration during R115777 therapy and that concurrent treatment with agents known to cause renal tubular injury be avoided. Minimal hematopoietic toxicity was observed in this trial, possibly due to the interrupted schedule.

Pharmacokinetic studies demonstrate that R115777 is orally bioavailable with plasma concentrations reaching those necessary for an antitumor effect in preclinical studies. Dose-proportional pharmacokinetics in the 25- to

325-mg dose range were noted for the oral solution throughout the 5-day dosing regimen. For the capsule, dose proportionality could be demonstrated in the 500- to 1,300-mg dose range after the first dose on day 1 but not after the last dose on day 6. Furthermore, the data suggest that the bioavailability of the capsules is less than that of the oral solution. However, because of the limited data and the high interindividual variability, more data are needed to investigate these observations. The pharmacokinetics of R115777 will be further explored in the drug development of R115777 to allow correlation of pharmacokinetic parameters with patient characteristics, disease state, liver function, and concomitant medication using population pharmacokinetic analysis techniques. Also, other drug formulations and the effect of food will be evaluated. Determination of the therapeutic level will allow assessment of the importance of the interindividual pharmacokinetic variability.

No objective tumor responses were observed in this phase I trial, although one patient with colon cancer metastatic to lungs experienced an improvement in her cough and decreased carcinoembryonic antigen levels. *ras* mutation analysis was performed on tumor specimens of patients participating in this trial. Three of 23 tumor specimens tested were positive for a *ras* mutation. The low frequency of *ras* mutations in this study can be attributed to the small sample size and patient selection factors. We also had other trials open at our institution for patients with known *ras* mutations, which may also have been a contributing factor. The results of phase II studies will be necessary to correlate *ras* mutation status with clinical response.

Although the protocol-defined maximum-tolerated dose was not achieved in this trial, analysis of toxicity data from all cycles and the pharmacokinetic data suggest that 500 mg bid for 5 days every 14 days is an appropriate dose for phase II studies. Pharmacokinetic studies demonstrate that 500 mg orally twice daily achieves plasma concentrations correlating with an antitumor effect in preclinical studies. The most frequent clinically significant adverse event related to R115777 was fatigue. Two of seven patients treated with 800 mg bid and four of six patients treated at 1,300 mg bid reported grade 2 worsening of performance status. All patients had advanced cancer and virtually all of them had received multiple previous therapies that could have contributed to declines in performance status. Nonetheless, the association of increasing frequency of significant performance status reduction with doses greater than 500 mg bid is reasonably strong. Clinical applications of this schedule might be in combination with cytotoxic chemotherapy, especially in light of the finding that cisplatin and paclitaxel have been demonstrated to have additive to synergistic effects when combined with farnesyl protein transferase inhibitors.[56] It remains to be seen whether the dose and schedule defined in this trial has utility as a chronic single-agent therapy, because only two patients received R115777 for at least 2 months. Further evaluation of this schedule can be addressed further in phase II studies with defined patient populations.

Further support for phase II testing with R115777 at the 500-mg orally, twice-daily schedule comes from pharmacokinetic data showing a possible deviation from dose proportionality for the oral bioavailability of R115777 at doses greater than 500 mg after repeated dosing. Our initial attempts to develop a surrogate biochemical correlate to monitor farnesyl protein transferase inhibition in peripheral-blood lymphocytes failed to detect changes that have been seen in cell culture studies. It remains to be determined whether the problem is associated with technical limitations of the assay or was due to the lack of protein turnover in the normally quiescent lymphocyte compartment.

In addition to the role mutant Ras may play in proliferation and apoptotic resistance in fibroblastic and epithelial malignancies,[57] Ras proteins also play a central role in both the activation[58] and apoptotic pathways[59] of normal T and natural killer cells. Maintenance of homeostasis in T-cell populations in adults involves a complex interplay of low-level proliferation of peripheral cells, replenishment by maturation of new naive cells from the thymus, and loss of cells by apoptosis.[60-63] The effect of inhibition of farnesyl protein transferase on T-cell homeostasis is unknown but an important concern in a chronically administered treatment. Natural killer cells, in contrast to T cells, are relatively short-lived, but little is known of the mechanisms regulating natural killer homeostasis. For these reasons, the effect of farnesyl protein transferase inhibitors on T-, B-, and natural killer–cell populations was assessed by flow cytometry. A small decrease in the total number of lymphocytes was noted at the end of the third cycle of therapy in this trial. Although this decrease was not of clinical significance, these data suggest the need for monitoring of the lymphocyte populations in long-term or continuous-treatment trials with R115777.

This study indicates that R115777 is orally bioavailable with an acceptable safety profile. This first clinical trial with a farnesyl protein transferase inhibitor suggests further investigations are warranted. Major challenges with this and other agents that are considered cytostatic are dose and schedule selection and sequencing in combination with other cancer treatments. Early clinical trials of these and other similar compounds should continue exploratory studies of potential novel surrogate end points of drug effect[64] (for example, levels of farnesylated proteins). These studies will help select optimal dosing schedules for definitive trials that would assess time to progression and, ultimately,

overall survival. It may be necessary to administer farnesyl protein transferase inhibitors chronically or in combination with other therapies for maximum clinical benefit. A phase I study of chronic dosing with R115777 suggests that myelosuppression occurs at significantly lower doses than has been observed in this phase I trial using an interrupted schedule.[65] The different toxicity profiles seen with the intermittent versus chronic dosing schedules have implications for sequencing with other agents that cause myelosuppression. Preclinical data with various farnesyl protein transferase inhibitors demonstrate synergy or additive effects with the traditional chemotherapy agents[56] and radiation therapy.[66] At the same time, preclinical data continue to underscore that *ras* (mutated or wild-type) is not the sole target of farnesyl transferase inhibition.[67] The fact that *ras* mutation status does not predict preclinical anticancer activity[28] suggests that phase II trials should target a wide variety of malignancies (including lung, breast, colon, ovarian, and hematologic malignancies), regardless of the incidence of *ras* mutations. Studies of continuous dosing and studies of R115777 in combination with other antineoplastic agents are ongoing.

ACKNOWLEDGMENT

The authors acknowledge Tanya Applegate, Caroline Fuery, Natalie Robert, Michele Steinmann, Jianbo Sun, and Jackie Toner for their contributions to the *ras* mutation analysis; Louise R. Finch, Christine Maloney, Annie Lennon-Gold, and Sylvia Avery for their research assistance; and David Venzon for the statistical review of the manuscript.

REFERENCES

1. Barbacid M: ras genes. Annu Rev Biochem 56:779-827, 1987
2. Grunewald K, Lyons J, Frohlich A, et al: High frequency of Ki-ras codon 12 mutations in pancreatic adenocarcinomas. Int J Cancer 43:1037-1041, 1989
3. Forrester K, Almoguera C, Han K, et al: Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature 327:298-303, 1987
4. Vogelstein B, Fearon ER, Hamilton SR, et al: Genetic alterations during colorectal-tumor development. N Engl J Med 319:525-532, 1988
5. Reynolds SH, Anna CK, Brown KC, et al: Activated protooncogenes in human lung tumors from smokers. Proc Natl Acad Sci USA 88:1085-1089, 1991
6. Knowles MA, Williamson M: Mutation of H-ras is infrequent in bladder cancer: Confirmation by single-strand conformation polymorphism analysis, designed restriction fragment length polymorphisms, and direct sequencing. Cancer Res 53:133-139, 1993
7. Almoguera C, Shibata D, Forrester K, et al: Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 53:549-554, 1988
8. Bos JL, Fearon ER, Hamilton SR, et al: Prevalence of ras gene mutations in human colorectal cancers. Nature 327:293-297, 1987
9. Mills NE, Fishman CL, Rom WN, et al: Increased prevalence of K-ras oncogene mutations in lung adenocarcinoma. Cancer Res 55: 1444-1447, 1995
10. Bos JL: ras oncogenes in human cancer: A review. Cancer Res 49:4682-4689, 1989 [published erratum appears in Cancer Res 50: 1352, 1990.
11. Moriyama N, Umeda T, Akaza H, et al: Expression of ras p21 oncogene product on human bladder tumors. Urol Int 44:260-263, 1989
12. Clark GJ, Der CJ: Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res Treat 35:133-144, 1995
13. Janes PW, Daly RJ, deFazio A, et al: Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. Oncogene 9:3601-3608, 1994
14. Patton SE, Martin ML, Nelsen LL, et al: Activation of the ras-mitogen-activated protein kinase pathway and phosphorylation of ets-2 at position threonine 72 in human ovarian cancer cell lines. Cancer Res 58:2253-2259, 1998
15. Feldkamp M, Lau N, Guha A: Astrocytomas are growth-inhibited by farnesyl transferase inhibitors through a combination of anti-proliferative and antiangiogenic activities. Proc Am Assoc Cancer Res 39:318, 1998 (abstr)
16. Guha A, Feldkamp MM, Lau N, et al: Proliferation of human malignant astrocytomas is dependent on Ras activation. Oncogene 15:2755-2765, 1997
17. Kato K, Der CJ, Buss JE: Prenoids and palmitate: Lipids that control the biological activity of Ras proteins. Semin Cancer Biol 3:179-188, 1992
18. Casey PJ, Solski PA, Der CJ, et al: p21ras is modified by a farnesyl isoprenoid. Proc Natl Acad Sci USA 86:8323-8327, 1989
19. Der CJ, Cox AD: Isoprenoid modification and plasma membrane association: Critical factors for ras oncogenicity. Cancer Cells 3:331-340, 1991
20. Jackson JH, Cochrane CG, Bourne JR, et al: Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. Proc Natl Acad Sci USA 87:3042-3046, 1990
21. Reiss Y, Goldstein JL, Seabra MC, et al: Inhibition of purified p21ras farnesyl:protein transferase by Cys-AAX tetrapeptides. Cell 62:81-88, 1990
22. Gutierrez L, Magee AI, Marshall CJ, et al: Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. EMBO J 8:1093-1098, 1989
23. Kato K, Cox AD, Hisaka MM, et al: Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc Natl Acad Sci USA 89:6403-6407, 1992
24. Kohl NE, Mosser SD, deSolms SJ, et al: Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. Science 260:1934-1937, 1993 (see comments)
25. James GL, Goldstein JL, Brown MS, et al: Benzodiazepine peptidomimetics: Potent inhibitors of Ras farnesylation in animal cells. Science 260:1937-1942, 1993 (see comments)
26. Garcia AM, Rowell C, Ackermann K, et al: Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. J Biol Chem 268:18415-18418, 1993
27. Ward R, Hawkins N, O'Grady R, et al: Restriction endonuclease-mediated selective polymerase chain reaction: A novel assay for the detection of K-ras mutations in clinical samples. Am J Pathol 153:373-379, 1998

28. Todd AV, Applegate TL, Fuery CJ, et al: Farnesyl transferase inhibitor (FTI): Effect of ras activation. Proc Am Assoc Cancer Res 39:317, 1998 (abstr)

29. Sepp-Lorenzino L, Ma Z, Rands E, et al: A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. Cancer Res 55:5302-5309, 1995

30. Smets G, Xhonneux B, Cornelissen F, et al: R115777, a selective farnesyl protein transferase inhibitor (FTI), induces anti-angiogenic, apoptotic and anti-proliferative activity in CAPAN-2 and LoVo tumor xenografts. Proc Am Assoc Cancer Res 39:318, 1998 (abstr)

31. Kohl NE, Wilson FR, Mosser SD, et al: Protein farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. Proc Natl Acad Sci USA 91:9141-9145, 1994

32. Kohl NE, Omer CA, Conner MW, et al: Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat Med 1:792-797, 1995 (see comments)

33. Mangues R, Corral T, Kohl NE, et al: Antitumor effect of a farnesyl protein transferase inhibitor in mammary and lymphoid tumors overexpressing N-ras in transgenic mice. Cancer Res 58:1253-1259, 1998

34. Sun J, Qian Y, Hamilton AD, et al: Ras CAAX peptidomimetic FTI 276 selectively blocks tumor growth in nude mice of a human lung carcinoma with K-Ras mutation and p53 deletion. Cancer Res 55:4243-4247, 1995

35. James GL, Goldstein JL, Brown MS: Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic in vitro. J Biol Chem 270:6221-6226, 1995

36. Yokoyama K, McGeady P, Gelb MH: Mammalian protein geranylgeranyltransferase-I: Substrate specificity, kinetic mechanism, metal requirements, and affinity labeling. Biochemistry 34:1344-1354, 1995 (published erratum appears in Biochemistry 34:14270), 1995

37. Whyte DB, Kirschmeier P, Hockenberry TN, et al: K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem 272:14459-14464, 1997

38. Rowell CA, Kowalczyk JJ, Lewis MD, et al: Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J Biol Chem 272:14093-14097, 1997

39. Liu M, Bryant MS, Chen J, et al: Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. Cancer Res 58:4947-4956, 1998

40. End D, Skrzat S, Devine A, et al: R115777, a novel imidazole farnesyl protein transferase inhibitor (FTI): Biochemical and cellular effects in H-ras and K-ras dominant systems. Proc Am Assoc Cancer Res 39:270, 1998

41. Armstrong SA, Hannah VC, Goldstein JL, et al: CAAX geranylgeranyl transferase transfers farnesyl as efficiently as geranylgeranyl to RhoB. J Biol Chem 270:7864-7868, 1995

42. Lebowitz PF, Casey PJ, Prendergast GC, et al: Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J Biol Chem 272:15591-15594, 1997

43. Tapon N, Hall A: Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 9:86-92, 1997

44. Du W, Lebowitz P, Prendergast G: Cell growth inhibition by farnesyltransferase inhibitors is mediated by gain of gernylgeranylated Rho B. Mol Cell Biol 19:1831-1840, 1999

45. Venet M, Angibaud P, Sanz G, et al: Synthesis and in-vitro structure-activity relationships of imidazolyl-2-quinolinones as farnesyl protein transferase inhibitors (FTI). Proc Am Assoc Cancer Res 39:318, 1998

46. Skrzat S, Angibaud P, Venet M, et al: R115777, a novel imidazole farnesyl protein transferase inhibitor (FTI) with potent oral antitumor activity. Proc Am Assoc Cancer Res 39:316, 1998 (abstr)

47. Miyake Y, Horiguchi M, Tomita N, et al: Occult macular dystrophy. Am J Ophthalmol 122:644-653, 1996

48. Pittler SJ, Fliesler SJ, Fisher PL, et al: In vivo requirement of protein prenylation for maintenance of retinal cytoarchitecture and photoreceptor structure. J Cell Biol 130:431-439, 1995

49. Christian MC, Korn EL: The limited precision of phase I trials. J Natl Cancer Inst 86:1662-1663, 1994 (editorial; comment)

50. Simon R, Freidlin B, Rubinstein L, et al: Accelerated titration designs for phase I clinical trials in oncology. J Natl Cancer Inst 89:1138-1147, 1997

51. Gibaldi M, Perier D: Pharmacokinetics. New York, NY, Marcel Dekker, Inc (1982)

52. Yan N, Ricca C, Fletcher J, et al: Farnesyltransferase inhibitors block the neurofibromatosis type I (NF1) malignant phenotype. Cancer Res 55:3569-3575, 1995

53. Nociari MM, Telford W, Russo C: Postthymic development of CD28− CD8+ T cell subset: Age-associated expansion and shift from memory to naive phenotype. J Immunol 162:3327-3335, 1999

54. d'Angeac AD, Monier S, Pilling D, et al: CD57+ T lymphocytes are derived from CD57− precursors by differentiation occurring in late immune responses. Eur J Immunol 24:1503-1511, 1994

55. Broers JL, Machiels BM, Kuijpers HJ, et al: A- and B-type lamins are differentially expressed in normal human tissues. Histochem Cell Biol 107:505-517, 1997

56. Moasser MM, Sepp-Lorenzino L, Kohl NE, et al: Farnesyl transferase inhibitors cause enhanced mitotic sensitivity to Taxol and epothilones. Proc Natl Acad Sci USA 95:1369-1374, 1998

57. Peli J, Schroter M, Rudaz C, et al: Oncogenic Ras inhibits Fas ligand-mediated apoptosis by downregulating the expression of Fas. Embo J 18:1824-1831, 1999

58. Gomez J, Gonzalez A, Martinez AC, et al: IL-2-induced cellular events. Crit Rev Immunol 18:185-220, 1998

59. Downward J: Ras signalling and apoptosis. Curr Opin Genet Dev 8:49-54, 1998

60. Mackall C, Hakim F, Gress R: T cell regeneration: All repertoires are not created equal. Immunol Today 18:245-251, 1997

61. Ho DD, Neumann AU, Perelson AS, et al: Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123-126, 1995 (see comments)

62. Hakim FT, Cepeda R, Kaimei S, et al: Constraints on CD4 recovery postchemotherapy in adults: Thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. Blood 90:3789-3798, 1997

63. Tanchot C, Rocha B: Peripheral selection of T cell repertoires: The role of continuous thymus output. J Exp Med 186:1099-1106, 1997

64. Gelmon KA, Eisenhaur EA, Harris AL, et al: Anticancer agents targeting signaling molecules and cancer cell environment: Challenges for drug development? J Natl Cancer Inst 91:1281-1287, 1999

65. Hudes G, Schol J, Baab J, et al: Phase I clinical and pharmacokinetic trial of the farnesyltransferase inhibitor R115777 on a 21-day dosing schedule. Proc Am Soc Clin Oncol 18:156a, 1999 (abstr)

66. Bernhard EJ, McKenna WG, Hamilton AD, et al: Inhibiting Ras prenylation increases the radiosensitivity of human tumor cell lines with activating mutations of ras oncogenes. Cancer Res 58:1754-1761, 1998

67. Prendergast GC, Davide JP, deSolms SJ, et al: Farnesyltransferase inhibition causes morphological reversion of ras-transformed cells by a complex mechanism that involves regulation of the actin cytoskeleton. Mol Cell Biol 14:4193-4202, 1994

Current status of clinical trials of farnesyltransferase inhibitors

Judith E. Karp, MD,* Scott H. Kaufmann, MD, PhD,† Alex A. Adjei, MD,† Jeffrey E. Lancet, MD,‡ John J. Wright, MD, PhD,§ and David W. End, PhD¶

Farnesyltransferase inhibitors represent a new class of agents that target signal transduction pathways responsible for the proliferation and survival of diverse malignant cell types. Although these agents were developed to prevent a processing step necessary for membrane attachment and maturation of Ras proteins, recent studies suggest that farnesyltransferase inhibitors block the farnesylation of additional cellular polypeptides, thereby exerting antitumor effects independent of the presence of activating *ras* gene mutations. Clinical trials of two farnesyltransferase inhibitors—the tricyclic SCH66336 and the methylquinolone R115777—as single agents have demonstrated disease stabilization or objective responses in 10 to 15% of patients with refractory malignancies. Combinations of farnesyltransferase inhibitors with cytotoxic chemotherapies are yielding complete and partial responses in patients with advanced solid tumors. A phase I trial of R115777 in refractory and relapsed acute leukemias induced responses in 8 (32%) of 25 patients with acute myelogenous leukemia (including two complete remissions) and in two of three with chronic myelogenous leukemia in blast crisis. In patients with solid tumors, accessible normal tissues such as peripheral blood lymphocytes or, perhaps more germane to epithelial malignancies, buccal mucosa have provided surrogate tissues that allow confirmation that farnesyltransferase is inhibited *in vivo* at clinically achievable drug doses. In conjunction with the R115777 acute leukemia trial, serial measurements provided evidence of farnesyltransferase enzyme inhibition, interference with farnesyltransferase function (*ie*, protein processing), and blockade of signal transduction pathways in leukemic bone marrow cells. Preclinical studies of farnesyltransferase inhibitor resistance and clinical trials of farnesyltransferase inhibitors in combination with other agents currently are in progress. Curr Opin Oncol 2001, 13:470–476

*University of Maryland Greenebaum Cancer Center, Baltimore, Maryland, USA; †Department of Oncology, Mayo Clinic, Rochester, Minnesota, USA; ‡University of Rochester Cancer Center, Rochester, New York, USA; §National Cancer Institute, Bethesda, Maryland, USA; and ¶Janssen Research Foundation, Titusville, New Jersey, USA.

Correspondence to Judith E. Karp, MD, University of Maryland Greenebaum Cancer Center, 22 S. Greene St., Room S9D07, Baltimore, MD 21201, USA; e-mail: jkarp@umm.edu

Current Opinion in Oncology 2001, 13:470–476

Abbreviations

AML	acute myelogenous leukemia
CML	chronic myelogenous leukemia
DLT	dose-limiting toxicity
NSCLC	nonsmall cell lung cancer
PTK	protein tyrosine kinase

ISSN 1040-8746

Molecular dissection of signal transduction pathways has not only provided a heightened understanding of the processes that govern cell proliferation, differentiation, and survival but also identified a panoply of potential therapeutic targets [1,2,3••]. Among these are the membrane-associated small GTPases encoded by the *ras* family of proto-oncogenes. These polypeptides are synthesized as cytosolic precursors that must attach to the cell membrane to transmit signals. Their membrane attachment usually depends upon the addition of a 15-carbon farnesyl group to a C-terminal amino acid sequence motif known as the CAAX box in a reaction catalyzed by the enzyme farnesyltransferase [4,5]. Farnesyltransferase inhibitors were developed on the premise that farnesyltransferase inhibition would prevent posttranslational Ras processing and, therefore, transduction of proliferative signals [3••,4–9]. However, subsequent studies suggest that the cytotoxic actions of farnesyltransferase inhibitors might also involve other farnesylated polypeptides, including Rho B [3••,6], components of the phosphoinositide 3-OH kinase (PI_3K)/Akt-2 pathway [10], and centromeric proteins that interact with microtubules to promote the completion of mitosis [11•].

Enhanced signaling by Ras proteins can result from two distinct processes: activating mutations or increased signaling from upstream molecules. Activating point mutations of *ras* genes occur in roughly 30% of all malignancies, with certain malignancies having a particularly high incidence. As a case in point, the incidence of *K-ras* mutations in pancreatic cancers approaches 90%. Mutations in H-*ras* and K-*ras* are detected in roughly 50% of all colorectal and nonsmall cell lung cancers (NSCLCs). Mutations and abnormal expression of *ras* genes, especially N-*ras*, are detected in diverse hematologic malignancies as well [12••]. Approximately 15 to 30% of all *de novo* acute myelogenous leukemias (AMLs), especially those arising in the setting of environmental or occupational exposures, exhibit N-*ras* mutations. Such mutations also occur in 10 to 15% of myelodysplasias, especially in chronic myelomonocytic leukemia (where the incidence exceeds 70%) and in cases of myelodysplasia that transform to AML. Likewise, N-*ras* and *K-ras* mutations are detected in roughly 40% of cases of newly-diagnosed multiple myeloma [13]. In addition to constitutive expression as a result of these mutations, Ras proteins are activated downstream of certain protein tyrosine kinases (PTKs, *eg*, growth factor receptors) [14].

This coupling of Ras with PTKs raises the possibility that PTK-driven malignancies may be susceptible to farnesyltransferase inhibitors. This possibility is particularly germane for malignancies where *ras* mutations are uncommon but PTK-driven signal transduction (*eg*, via epidermal growth factor receptors) is crucial to cancer cell growth and survival, as in the case of breast cancers.

There are a growing number of farnesyltransferase inhibitors in varying stages of preclinical and clinical development [4,9,15,16]. Different structural classes include CAAX peptidomimetics, the nonpeptide peptidomimetics, farnesyl diphosphate analogues, and bisubstrate inhibitors, all of which inhibit the farnesyltransferase enzyme selectively and competitively. Phase I and II clinical trials of diverse farnesyltransferase inhibitors, alone or in combination with traditional cytotoxic chemotherapeutic agents, are being conducted in the United States and Europe for a broad spectrum of malignancies. This article focuses on those farnesyltransferase inhibitors with the greatest amount of clinical testing to date, the nonpeptide peptidomimetic farnesyltransferase inhibitors SCH66336 (Schering-Plough Corp., Kenilworth, NJ) and R115777 (Janssen Research Foundation, Titusville, NJ). The tricyclic SCH66336 targets H-Ras and to a lesser extent K-Ras [17,18], whereas the methylquinolone R115777 exerts its activities against farnesylation of N-Ras and other Ras proteins [4,19••].

Clinical trials in solid tumors

Single agent trials

Phase I trials of SCH66336 and R115777 as single oral agents have defined pharmacokinetic and toxicity profiles. These agents display disparate pharmacokinetics but share important similarities in dose-limiting toxicities (DLTs) and clinical responses. Adjei *et al.* [20••] administered escalating doses of SCH66336 for 7 days every 3 weeks in 20 patients with advanced solid tumors. DLT at 400 mg twice daily consisted of grade 4 gastrointestinal toxicities and transient creatinine elevations related to dehydration, leading to a recommended phase II dose of 350 mg twice daily. One patient with metastatic NSCLC achieved an objective partial response, and an additional eight patients experienced disease stabilization lasting for as many as 10 cycles of drug. Eskens *et al.* [21] administered SCH66336 on a continuous basis at doses ranging from 25 mg twice daily to 400 mg twice daily and observed transient and reversible dose-limiting myelosuppression and gastrointestinal, neurocortical, and renal toxicities at 300 mg twice daily. The occurrence of DLTs at the lower dose of 300 mg twice daily relative to the 7 day schedule likely relates to the pharmacokinetic findings of a greater than dose-proportional increase in drug exposure and peak plasma concentrations with the continuous dosing schedule. On the basis of pharmacokinetic and toxicity data, the recommended dose for phase II studies using continuous oral dosing is 200 mg twice daily. Similar pharmacokinetic and toxicity data were obtained in studies using a 2 weeks on, 2 weeks off schedule [22], again demonstrating drug accumulation over the 2-week period.

Phase I trials of R115777 in patients with advanced solid tumors have examined a variety of dosing schedules, including twice daily for 5 days every 2 weeks [23], twice daily for 21 days every 4 weeks [24], and continuous twice daily dosing [25]. As with SCH66336, DLTs consisted of dose-related, reversible myelosuppression (particularly with the more prolonged dose schedules), neurologic changes (both central and peripheral), and gastrointestinal and renal dysfunction. Fatigue, hyperbilirubinemia, and skin rashes also were detected, particularly with 21-day and continuous dosing schedules. Disease stabilization lasting longer than 5 months, associated with an approximately 50% drop in carcinoembryonic antigen serum levels, was observed in one patient with metastatic colon cancer in a study by Zujewski *et al.* [23] of 26 patients receiving R115777 for 5 days every 21 days. Hudes *et al.* [24] noted disease stabilization of at least 6 months in three of 22 patients treated with the 21-day dosing schedule. Of 16 patients who received continuous dosing, one with NSCLC achieved a partial response, and two with colorectal cancers had disease stabilization with at least 50% decreases in carcinoembryonic antigen serum levels [25]. Pharmacokinetic studies demonstrated that R11577 levels exhibit dose proportionality without significant drug accumulation or cumulative toxicities [23–25]. The phase II doses recommended on the basis of these phase I trials are 500 mg twice daily for 5 days every 2 weeks for the intermittent schedule and 300 mg twice daily for prolonged (≥ 21-day) administration.

A single phase II trial of R115777 has been reported in abstract form [26]. Administration of R115777 at 300 mg twice daily for 21 days every 4 weeks to 27 women with advanced breast cancer resulted in confirmed partial responses in three patients (12%), with responses documented in liver, lung, nodes, and skin sites. Disease stabilization was observed in an additional nine (35%). The major toxicity was grade 3 to 4 self-limited myelosuppression in roughly 30%, with lesser grades of paresthesias, diarrhea, skin rash, and fatigue in 10 to 30% of patients.

Combination trials

Preclinical studies have demonstrated that farnesyltransferase inhibitors exhibit synergistic effects when combined with taxanes [27,28] or, in some contexts, platinating agents [29•]. Additive effects have been observed when these agents are combined with gemcitabine [29•]. Based on these results, combinations of both SCH66336 and R115777 with cytotoxic chemotherapies are being

explored in patients with refractory solid tumors, with exciting results.

Results of several phase I studies combining SCH66336 with other agents have been reported in preliminary form. A phase I trial of SCH66336 plus gemcitabine [30] yielded partial responses in two of nine pancreatic cancers, minor responses in pancreatic cancer and mesothelioma, and long-term (> 6 months) disease stabilization in an additional 11 (44%) of 25 patients. Phase II testing will proceed with the combination of daily oral SCH66336 at 200 mg twice daily with weekly gemcitabine 1,000 mg/m^2. The combination of SCH66336 as much as 150 mg twice daily and paclitaxel as much as 175 mg/m^2 [31] has produced a striking 40% response rate in the first 10 patients, with responses seen in both treatment-naive patients and patients previously heavily treated, particularly patients with NSCLC, and including those who have received taxanes.

R115777 is being combined with diverse antitumor agents including trastuzumab (Herceptin; Genentech, Inc., S. San Francisco, CA) [32]; gemcitabine alone [33] or with cisplatin [34]; docetaxel [35]; and capecitabine, the oral prodrug of 5-fluorouracil [36]. These studies extend the provocative results detected with the initial phase I trials of R115777 as a single agent. For example, the combination of R115777 on days 1 to 14 with gemcitabine 1,000 mg/m^2 on days 1 and 8 plus cisplatin 75 mg/m^2 on day 1 has induced responses in 33% (five of 15, four partial responses and one complete remission [CR]) of evaluable patients, with R115777 as much as 300 mg twice daily tested to date [34]. Similar results have been observed in 24 patients receiving R115777 at 200 mg twice daily plus docetaxel 75 mg/m^2 every 21 days, with one (4%) CR in breast cancer, four (17%) partial responses in breast and lung cancers, and six (25%) disease stabilizations with a decrease of more than 50% in the levels of tumor-associated markers [35].

To date, combination studies have demonstrated that SCH66336 and R115777 do not alter the pharmacokinetics or exacerbate the toxicities of the chemotherapeutic agents or trastuzumab. Studies that continue to define optimal combinations, doses, and schedules of farnesyltransferase inhibitors with mechanistically complementary agents should remain a high priority for the overall clinical development of farnesyltransferase inhibitors.

Clinical trials in hematologic malignancies

Hematologic malignancies present a fertile testing ground for agents that target signal transduction pathways, including (but certainly not limited to) Ras-based signaling, for several reasons. First, as noted above, a substantial proportion of AMLs, myelodysplasias, and multiple myelomas have activating *ras* mutations. Second, many hematologic malignancies evince constitutive activation of growth factor receptor PTKs, caused by either point mutations or translocations of receptor-encoding genes. This receptor PTK activation leads in turn to deregulation of Ras activity. Moreover, Ras activity can be triggered by cytoplasmic PTKs, as exemplified by the BCR-ABL fusion protein that typifies chronic myelogenous leukemia (CML). Lastly, even in the absence of *ras* or PTK activating gene mutations or translocations, net Ras activity could be driven by high concentrations of locally active growth and survival factors. In this regard, vascular endothelial growth factor may be produced by the malignant cell or by the surrounding stromal cells or both, leading to ligand-receptor binding with PTK activation and downstream Ras signaling activity through autocrine and paracrine mechanisms [37]. To the extent that farnesyltransferase inhibitors target Ras signaling pathways, these observations suggest that farnesyltransferase inhibitors are reasonable agents to test in hematologic malignancies.

In a phase I trial in adults with acute leukemias and CML in blastic crisis [12••], R115777 was administered to 34 patients (median age 65 y, range 24–77 y) twice daily for as many as 21 days and continued for as many as three additional 21-day cycles if response or disease stabilization was observed. DLT, which consisted of ataxia and confusion, occurred at 1,200 mg twice daily. At 900 mg twice daily, transient toxicities occurred in six (55%) of 11 patients, including less than grade 2 thirst, creatinine elevations, and paresthesias. Reversible myelosuppression occurred at doses of 600 mg twice daily and higher. Responses were observed in eight (32%) of 25 patients with AML, including two CRs, and in two (67%) of three patients with CML in blast crisis. These responses occurred across all dose levels. In contrast, no responses were seen in six patients with acute lymphoblastic leukemia, including three with the Philadelphia chromosome. Interestingly, no *ras* mutations were detected in any leukemias in this study. In summary, R115777 induced significant clinical responses in patients with resistant disease without major toxicities, even in older adults. These results lay the foundation for phase II testing of R115777 in high-risk AMLs and myelodysplasias at a dose of 600 mg twice daily for 21 days.

Because Ras-driven signal transduction might play a role in the pathogenesis and progression of multiple myeloma [13], there has been considerable interest in testing farnesyltransferase inhibitors in this disease. *In vitro* studies have demonstrated that the peptidomimetic farnesyltransferase inhibitor-277 has activity against all multiple myeloma cell lines tested, especially those with N-*ras* mutations, with antitumor activity being independent of resistance to doxorubicin or melphalan [38]. A phase II trial evaluating the efficacy and safety of R115777 is underway in patients with refractory and relapsed multiple

myeloma, with the selected dose at 300 mg twice daily to mitigate against any myelosuppression (W.S. Dalton, MD, PhD, oral communication).

Biologic markers of farnesyltransferase inhibitor effects

A pivotal component of clinical trials with new agents is the demonstration that the presumptive molecular target is affected by the agent under study. In studies of farnesyltransferase inhibitors, it is critical to determine whether farnesyltransferase is inhibited *in vivo* in target tumor cells or in reliable surrogate tissues. Potential markers of the effects of farnesyltransferase inhibitors on the process of farnesylation include direct farnesyltransferase enzyme inhibition, interference with farnesyltransferase function (*ie*, blockade of protein farnesylation with accumulation of the unprocessed protein precursor), interruption of signal transduction pathways measured by lack of phosphorylation of crucial intermediates (*eg*, extracellular signal-related kinases as effector enzymes downstream of Ras) or lack of signal-driven gene transcription (*eg*, vascular endothelial growth factor), and quantification of intracellular drug levels.

The ease of assay performance and potential usefulness of diverse markers have been evaluated in a variety of preclinical studies. In some cases, direct measurement of farnesyltransferase activity as a marker of farnesyltransferase action is problematic, for two reasons. First, farnesyltransferase activity can be difficult to assay in extracts prepared from some tissues, apparently because of endogenous inhibitors [39]. Second, because farnesyltransferase inhibitors are reversible enzyme inhibitors, dissociation of these agents from the target enzyme caused by the dilution involved in preparing cellular extract can, in turn, result in underestimation of the degree of farnesyltransferase inhibition. To circumvent these problems, many studies have focused on measuring farnesyltransferase inhibition by detecting altered farnesylation of farnesyltransferase substrates. Because there are significant difficulties in detecting Ras proteins in human tumor specimens, and because both N-Ras and K-Ras can undergo alternative prenylation [40••], most efforts to detect inhibition of substrate farnesylation have focused on other polypeptides. Studies by Sinensky *et al.* [41] indicated that lamin A, a structural component of the nuclear envelope, is a convenient marker for farnesyltransferase inhibition because the proteolytic removal of the C-terminal 13 amino acids from the lamin A precursor is absolutely dependent upon previous prenylation. More recent studies have identified other markers that undergo a shift in mobility when prenylation is inhibited, including the chaperone protein HDJ-2 [42,43] and the peroxisomal protein PxF [44]. Comparison of these potential markers in human tumor cell lines revealed that unprocessed HDJ-2 and prelamin A were readily detected when either cycling or noncycling cells were treated *in vitro* with SCH66336, farnesyltransferase inhibitor-277, or R115777 [40••; A.A. Adjei, MD, PhD, oral communication).

These markers have been used subsequently to confirm that farnesyltransferase is inhibited at therapeutically achievable farnesyltransferase inhibitor concentrations. In instances where it is impractical or unfeasible to obtain serial tumor biopsies, accessible normal tissues have been examined. Many investigators have used peripheral blood lymphocytes for this purpose. A phase I study of R115777 in children with solid tumors and neurofibromatosis (a heritable disorder characterized by net Ras overexpression caused by loss of the *NF-1* gene encoding Ras-glutamyl transpeptidase, the enzyme responsible for Ras inactivation) has demonstrated a decrease in HDJ-2 farnesylation at doses of 200 mg/m^2 twice daily for 21 days [45]. Likewise, inhibition of HDJ-2 farnesylation ranging from 14 to 47% of baseline values has been detected in peripheral blood lymphocytes obtained from adults with advanced solid tumors during a phase I trial combining R115777 and capecitabine, with enzyme inhibition occurring at all dose levels of R115777 tested (100–300 mg twice daily) [36].

However, peripheral blood lymphocytes may differ from epithelial cells in many important ways, including the expression of specific farnesylated proteins such as nuclear membrane lamins. Adjei *et al.* [40••] used buccal mucosa as a readily accessible epithelial tissue in which the effects of SCH66336 on farnesyltransferase substrates could be examined. Results of this analysis revealed that SCH66336 inhibited prelamin A processing in a dose-dependent manner.

More recent studies have attempted to confirm that farnesyltransferase inhibitors actually inhibit farnesylation in tumor tissue. In a phase IB trial of SCH66336 administered preoperatively to previously untreated patients with squamous cell carcinoma of head and neck, Kies *et al.* [46] observed farnesyltransferase inhibition at all dose levels, as measured in surgical biopsy specimens by an 11 to 50% increase in the unfarnesylated form of HDJ-2. It is important to stress, however, that other solid tumors are likely to be less amenable to the repeated biopsies required to perform this type of serial analysis.

By contrast, hematologic malignancies offer an ideal setting in which to make serial measurements directly in the malignant cell cohort, in large part because of the accessibility of the target cell population. In this regard, the phase I trial of R115777 in acute leukemia provided the first evidence for successful inhibition of farnesyltransferase in neoplastic cells *in vivo* [12••]. Serial measurements performed on target leukemic bone marrow cells throughout R115777 administration demonstrated that

(1) Farnesyltransferase enzyme inhibition occurred at doses of 300 mg twice daily or more
(2) At doses of at least 600 mg twice daily, R115777 blocked farnesylation of certain cellular proteins that require this biochemical modification for full activity (nuclear membrane lamin A and the heat shock chaperone protein HDJ-2)
(3) In cells with constitutive Ras signaling as evidenced by extracellular signal-related kinase phosphorylation (activation), phosphorylated extracellular signal-related kinases became undetectable after R115777 administration
(4) R115777 accumulated in the bone marrow compartment in a dose-dependent manner and reached levels equal to greater than peak plasma concentrations (C_{max}) that were sustained throughout the period of drug administration

Resistance to farnesyltransferase inhibitors

Intrinsic and acquired resistance of neoplastic cells to anticancer agents is a major factor underlying the limited efficacy of cancer therapeutic regimens. Thus, elucidating resistance mechanisms and designing ways of circumventing these mechanisms are important tactics in novel drug development. A few reports on resistance to the farnesyltransferase inhibitors have been published. First, a human farnesyltransferase mutant, Y361L, has been genetically engineered [47]. This Y361L mutant with an amino acid change at residue 361 of the β subunit exhibits increased resistance to farnesyltransferase inhibitors, particularly the tricyclic compounds, which are nonpeptide peptidomimetics. Interestingly, this mutant exhibited an increased affinity for peptides terminating with CIIL, a motif recognized by geranylgeranyltransferase I. Whether similar mutations occur in the clinical setting remains to be established.

A second mechanism of resistance appears to involve selective adaptation of cells to farnesyltransferase inhibitors. Prendergast *et al.* [48] described a cell line that displayed resistance to the farnesyltransferase inhibitor L-739,749. Reduced inhibition of farnesyltransferase activity and farnesylation-dependent protein processing was detected in intact cells but not in cell lysates, raising the possibility that the cells had diminished farnesyltransferase inhibitor uptake. Whether a similar mechanism of resistance will be observed with farnesyltransferase inhibitors being tested clinically remains to be seen.

A third mechanism of resistance involves activation of the PI_3K/Akt survival pathway and has been shown to confer resistance to farnesyltransferase inhibitors. Overexpression of the antiapoptotic Bcl-2 family member Bcl-X_L blocks farnesyltransferase inhibitor-induced apoptosis in NIH 3T3 cells [10]. Likewise, constitutively activated Akt can block the proapoptotic effects of farnesyltransferase inhibitors in these cells [10]. Akt-induced phosphorylation of the proapoptotic Bcl-2 family member BAD appears to link these observations: when BAD is phosphorylated by Akt, it cannot bind to and inhibit Bcl-X_L. Thus, activation of a pathway that involves PI_3K, Akt, and BAD results in less opposition to the antiapoptotic effects of Bcl-X_L. Because this is a generic pathway that inhibits apoptosis induced by a variety of agents, cells that are resistant to farnesyltransferase inhibitors based on this mechanism are predicted to be cross-resistant to a wide range of anticancer drugs. In addition, this pathway has been established using cell lines transfected with genes encoding specific proteins. Polypeptides are commonly expressed at extremely high levels in these transfection experiments. Further studies are required to determine whether this mechanism contributes to resistance encountered when patients are treated with farnesyltransferase inhibitors in the clinic.

Conclusions

The farnesyltransferase inhibitors are signal transduction inhibitors that display promising clinical activity against a broad spectrum of malignancies. On the molecular level, it is clear that farnesyltransferase inhibitors target multiple mechanisms of cellular survival: for example, angiogenesis and inhibitors of apoptosis [19••,37,49–51,52•]. The inhibition of angiogenesis is caused, at least in part, by the ability of farnesyltransferase inhibitors to block vascular endothelial growth factor production by tumor cells and endothelial cells in response to hypoxia or other stimuli that trigger the PI_3K pathway [10,19••,50,51]. Farnesyltransferase inhibitor-induced apoptosis reflects several mechanisms, including the ability of farnesyltransferase inhibitors to prevent PI_3K-driven phosphorylation and inactivation of the proapoptotic BAD protein [10]. In addition, farnesyltransferase inhibitors disrupt orderly cell cycle progression, in part through interference with the farnesylation and function of the centromere proteins centromeric protein-E and centromeric protein-F, which are critical for normal mitosis [11•], and in part through p53-dependent and p53-independent cell cycle arrest at both G_1S and G_2M checkpoints [49]. These effects can occur in tumor cells without *ras* mutations but with net Ras activation triggered in part by constitutive PTK expression, *eg*, astrocytoma cells [50], murine and human CML cells expressing the p210 BCR-ABL protein [52•], and human p190 BCR-ABL–positive acute lymphoblastic leukemia [53]. The ability of farnesyltransferase inhibitors to exert antitumor effects at least partly independent of *ras* mutations extends their clinical applicability to the vast majority of cancers.

What are the goals for continuing clinical development of farnesyltransferase inhibitors? The ability to use farnesyltransferase inhibitors with traditional cytotoxic drugs or immune-based agents without incurring pharmacokinetic alterations or synergistic toxicities should allow

combinations that selectively target and modulate nonoverlapping pathways. The clinical testing of such combinations is already underway, as exemplified by the studies discussed. Although these studies are being conducted in established, irreversible malignancies, farnesyltransferase inhibitors should be considered in two additional contexts. First, in light of their activity in refractory or relapsed AML, farnesyltransferase inhibitors should be examined in the minimal residual disease state (*eg*, AML in remission). Second, the ability of farnesyltransferase inhibitors to prevent the progression of clonal disorders from premalignant conditions to fully-transformed states should be studied. Several hematologic malignancies might be appropriate diseases in which to test this latter concept: for instance, high-risk myelodysplasia and monoclonal gammopathy of uncertain or borderline significance, which have defined rates of progression to full-blown AML and multiple myeloma, respectively. It is possible to extend this concept to epithelial cancers as well by testing the ability of farnesyltransferase inhibitors to interdict the progression of premalignant cells or cells with limited invasive potential (*eg*, carcinomas *in situ*) into fully invasive and disseminated cancers. The clinical trials currently in progress will provide the critical foundations for defining the optimal roles of farnesyltransferase inhibitors in the chemotherapy and chemoprevention of cancer.

Acknowledgments

This work was supported by National Cancer Institute Cooperative Agreement U01 CA69854 (J.E.K.) and a grant from Janssen Research Foundation (J.E.K.).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • Of special interest
- •• Of outstanding interest

1 Ito T, May WS: Drug development train gathering steam. Nat Med 1996, 2:403–404.

2 McCubrey JA, May WS, Duronio V, et al.: Serine/threonine phosphorylation in cytokine signal transduction. Leukemia 2000, 14:9–21.

3 • Reuter CWM, Morgan MA, Bergmann L: Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? Blood 2000, 96:1655–1669.
Outstanding review of the role of Ras in the pathogenesis of hematologic malignancies and of agents that target Ras and downstream components of Ras signaling pathways. Focus is on preclinical aspects of drug development (molecular mechanisms, animal models).

4 End DW: Farnesyl protein transferase inhibitors and other therapies targeting the Ras signal transduction pathway. Invest New Drugs 1999, 17:241–258.

5 Reiss Y, Goldstein JL, Seabra MC, et al.: Inhibition of purified Ras farnesyl: protein transferase by Cys-AAX peptides. Cell 1990, 62:81–88.

6 Du W, Lebowitz PF, Prendergast GC: Cell growth inhibition by farnesyltransferase inhibitors is mediated by a gain of geranylgeranylated Rho B. Mol Cell Biol 1999, 19:1831–1840.

7 Gibbs JB, Oliff A: The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. Ann Rev Pharmacol Toxicol 1997, 37:143–166.

8 James GL, Goldstein JL, Brown MS, et al.: Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. Science 1993, 260:1937–1942.

9 Rowinsky EK, Windle JJ, Von Hoff DD: Ras protein farnesyltransferase: a strategic target for anticancer therapeutic development. J Clin Oncol 1999, 17:3631–3652.

10 Jiang K, Coppola D, Crespo NC, et al.: The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. Mol Cel Biol 2000, 20:139–148.

11 • Ashar HR, James L, Gray K, et al.: Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with microtubules. J Biol Chem 2000, 275:30451–30457.
Defines a new intracellular target for farnesyltransferase inhibitor action. Inhibition of farnesylation of the centromeric proteins alters the normal interaction between centromeres and microtubules, causes cell cycle arrest at the G_2M checkpoint, and disrupts orderly mitosis.

12 •• Karp JE, Lancet JE, Kaufmann SH, et al.: Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase I clinical-laboratory correlative trial. Blood 2001, 97:3361–3369.
The first clinical trial of farnesyltransferase inhibitor in leukemias, and the first trial to demonstrate farnesyltransferase inhibitor-induced changes in multiple parameters of enzyme activity and function in the target malignant cell population.

13 Hallek M, Bergsagel PL, Anderson KC: Multiple myeloma: increasing evidence for a multistep transformation process. Blood 1998, 91:3–21.

14 Sasaoka T, Langlois W, Leitner JW, et al.: The signaling pathway coupling epidermal growth factor receptors to activation of p21^{ras}. J Biol Chem 1994, 51:32621–23625.

15 Adjei AA: Protein farnesyl transferase as a target for the development of anticancer agents. Drugs Future 2000, 25:1069–1079.

16 Gelb MH, Scholten JD, Sebolt-Leopold JS: Protein prenylation: from discovery to prospects for cancer treatment. Curr Opin Chem Biol 1998, 2:40–48.

17 Liu M, Bryant MS, Chen J, et al.: Antitumor activity of SCH66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human xenograft models and wap-ras transgenic mice. Cancer Res 1998, 58:4947–4956.

18 Njoroge FG, Taveras AG, Kelly J, et al.: (+)-4-[2-[4-(8-Chloro-3,10-dibromo-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]-pyridin-11(R)-yl)-1-piperidinyl]-2-oxo-ethyl]-1-piperidinecarboxamide (SCH-66336): a very potent farnesyl protein transferase inhibitor as a novel antitumor agent. J Med Chem 1998, 41:4890–4902.

19 •• End DW, Smets G, Todd AV, et al.: Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 in vivo and in vitro. Cancer Res 2001, 61:131–137.
R115777 exhibits preferential inhibition of N-Ras farnesylation and multiple mechanisms of action, including antiangiogenesis, antiproliferative, and prospoptotic effects against diverse human tumor cell lines in vitro and in animal xenograft models.

20 •• Adjei AA, Erlichman C, Davis JN, et al.: A phase I trial of the farnesyl transferase inhibitor SCH66336: evidence for biologic and clinical activity. Cancer Res 2000, 60:1871–1877.
The first phase I clinical trial of SCH66336 in solid tumors, and the first study to use buccal mucosa as a reliable surrogate tissue to measure SCH66336-induced changes in the farnesylation of the precursor protein prelamin A as a marker of drug effect.

21 Eskens FALM, Awada A, Cutler DL, et al.: Phase I and pharmacokinetic study of the oral farnesyl transferase inhibitor SCH 66336 given twice daily to patients with advanced solid tumors. J Clin Oncol 2001, 19:1167–1175.

22 Hurwitz HI, Colvin OM, Petros WP, et al.: Phase I and pharmacokinetic study of SCH66336, a novel FPTI, using a 2-week on, 2-week off schedule [abstract]. Proc Am Soc Clin Oncol 1999, 18:156a.

23 Zujewski J, Horak ID, Bol CJ, et al.: Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. J Clin Oncol 2000, 18:927–941.

24 Hudes G, Scholl J, Baab J, et al.: Phase I clinical and pharmacokinetic trials of the farnesyltransferase inhibitor R115777 in advanced cancer [abstract]. Proc Am Soc Clin Oncol 1999, 18:156a.

25 Schellens JH, de Klerk G, Swart M, et al.: Phase I and pharmacologic study of the novel farnesyltransferase inhibitor (FTI) R115777 [abstract]. Proc Am Soc Clin Oncol 2000, 19:184a.

26 Johnston S, Ellis P, Houston S, et al.: A phase II study of the farnesyltransferase inhibitor R115777 in patients with advanced breast cancer [abstract]. Proc Am Soc Clin Oncol 2000, 19:93a.

27 Moasser MM, Sepp-Lorenzino L, Kohl NE, et al.: Farnesyltransferase inhibitors cause enhanced mitotic sensitivity to taxol and epothelones. Proc Natl Acad Sci U S A 1998, 95:1369–1374.

28 Shi B, Yaremko B, Hajian G, et al.: The farnesyl protein transferase inhibitor SCH66336 synergizes with taxanes in vitro and enhances their antitumor activity in vivo. Cancer Chemother Pharmacol 2000, 46:387–393.

29 Adjei AA, Davis JN, Erlichman C, et al.: Synergy of the protein farnesyltransferase inhibitor SCH66336 and cisplatin in human cancer cell lines. Clin Cancer Res 2001, 7, in press.
Careful in vitro synergy studies that form the basis for phase I testing of SCH66336 in combination with platinating agents.

30 Hurwitz H, Amado R, Prager D, et al.: Phase I pharmacokinetic trial of the farnesyltransferase inhibitor SCH66336 plus gemcitabine in advanced cancers (abstract). Proc Am Soc Clin Oncol 2000, 19:185a.

31 Khuri F, Glisson B, Meyers M, et al.: Phase I study of farnesyltransferase inhibitor (FTI) SCH66336 with paclitaxel in solid tumors: dose finding, pharmacokinetics, efficacy/safety (abstract). Proc Am Soc Clin Oncol 2000, 19:205a.

32 Schwartz G, Rowinsky EK, Rha SY, et al.: A phase I, pharmacokinetic, and biologic correlative study of R115777 and trastuzumab (Herceptin) in patients with advanced cancer (abstract). Proc Am Soc Clin Oncol 2001, 20:81a.

33 Patnaik A, Eckhardt S, Ibzicka E, et al.: A phase I and pharmacokinetic (PK) study of the farnesyltransferase inhibitor R115777 in combination with gemcitabine (Gem) (abstract). Proc Am Soc Clin Oncol 2000, 19:2a.

34 Adjei AA, Erlichman C, Marks RS, et al.: A phase I trial of the farnesyltransferase inhibitor R115777, in combination with gemcitabine and cisplatin in patients with advanced cancer (abstract). Proc Am Soc Clin Oncol 2001, 20:81a.

35 Piccart-Gebhart MJ, Branle F, deValeriola D, et al.: A phase I, clinical and pharmacokinetic (PK) trial of the farnesyltransferase inhibitor (FTI) R115777 + Docetaxel: a promising combination in patients with solid tumors (abstract). Proc Am Soc Clin Oncol 2001, 20:80a.

36 Holden SN, Eckhardt S, Fisher S, et al.: A phase I pharmacokinetic and biologic study of the farnesyltransferase inhibitor (FTI) R115777 and capecitabine in patients with advanced solid malignancies. Proc Am Soc Clin Oncol 2001, 20:80a.

37 Chin L, Tam A, Pomerantz J, et al.: Essential role for oncogenic Ras I tumor maintenance. Nature 1999, 400:468–472.

38 Bolick SCE, Campbell K, Hamilton A, et al.: Myeloma cells with activated N-Ras are sensitive to FTI-277. Proc Am Assoc Cancer Res 1999, 40:832.

39 James GL, Brown MS, Goldstein JL: Assays for inhibitors of CAAX farnesyltransferase in vitro and in intact cells. Methods Enzymol 1995, 255:38–46.

40 Adjei AA, Davis JN, Ehrlichman C, et al.: Comparison of potential markers of farnesyltransferase inhibition. Clin Cancer Res 2000, 6:2318–2325.
Landmark work defining farnesyltransferase inhibitor-induced changes in intracellular farnesylation of unprocessed prelamin A and the chaperone protein HDJ-2 as reproducible markers of drug effect, independent of cell cycle status. Assays established in human tumor cell lines.

41 Sinensky M, Fantle K, Dalton M: An antibody which specifically recognizes prelamin A but not mature lamin A: application to detection of blocks in farnesylation-dependent protein processing. Cancer Res 1994, 54:3229–3232.

42 Britten CD, Rowinsky E, Yao S-L, et al.: The farnesyl protein transferase (FPTase) inhibitor L-778,123 in patients with solid cancers [abstract]. Proc Am Soc Clin Oncol 1999, 18:155a.

43 Neckers L, Mimnaugh E, Schulte TW: The Hsp90 chaperone family. In Stress Proteins. Edited by Latchman DS. New York: Springer-Verlag; 1999:9–42.

44 James GL, Goldstein JL, Pathak RK, et al.: PxF, a prenylated protein of peroxisomes. J Biol Chem 1994, 269:14182–14190.

45 Widemann BC, Salzer WL, Arceci RJ, et al.: Phase I trial of R115777, an oral farnesyltransferase (Ftase) inhibitor, in children with refractory solid tumors and neurofibromatosis type I (NF1) [abstract]. Proc Am Soc Clin Oncol 2001, 20:368a.

46 Kies MS, Clayman GL, El-Naggar AK, et al.: Induction therapy with SCH66336, a farnesyltransferase inhibitor, in squamous cell carcinoma (SCC) of the head and neck [abstract]. Proc Am Soc Clin Oncol 2001, 20:225a.

47 Del Villar K, Urano J, Guo L, et al.: A mutant form of human protein farnesyltransferase exhibits increased resistance to farnesyltransferase inhibitors. J Biol Chem 1999, 274:27010–27017.

48 Prendergast GC, Davide JP, Lebowitz PF, et al.: Resistance of a variant ras-transformed cell line to phenotypic reversion by farnesyltransferase inhibitors. Cancer Res 1996, 56:2626–2632.

49 Ashar HR, James L, Gray K, et al.: The farnesyltransferase inhibitor SCH 66336 induces a $G_2 \rightarrow M$ or G_1 pause in sensitive human tumor cell lines. Exp Cell Res 2001, 262:17–27.

50 Feldkamp MM, Lau N, Guha A: Growth inhibition of astrocytoma cells by farnesyl transferase inhibitors is mediated by a combination of antiproliferative, pro-apoptotic and anti-angiogenic effects. Oncogene 1999, 18:7514–7526.

51 Gu WZ, Tahir YC, Wang HC, et al.: Effect of novel CAAX peptidomimetic farnesyltransferase inhibitor on angiogenesis in vitro and in vivo. Eur J Cancer 1999, 35:1394–1401.

52 Peters DG, Hoover RR, Gerlach MJ, et al.: Activity of the farnesyl protein transferase inhibitor SCH66336 against BCR/ABL-induced murine leukemia and primary cells from patients with chronic myeloid leukemia. Blood 2001, 97:1404–1412.
Demonstration of farnesyltransferase inhibitor activity in murine and primary human CML cells. The activity extends beyond inhibition of Ras protein activity, in particular inducing G_2M blockade by preventing centromeric protein farnesylation.

53 Reichert A, Heisterkamp N, Daley GQ, et al.: Treatment of Bcr/Abl-positive acute lymphoblastic leukemia in P190 transgenic mice with the farnesyl transferase inhibitor SCH66336. Blood 2001, 97:1399–1403.